PREPARATION AND PHARMACOLOGICAL EVALUATION OF A POLYHERBAL PREPARATION (POL-6) ON ALCOHOL DEPRIVATION EFFECTS FOLLOWING VOLUNTARY ALCOHOL CONSUMPTION IN RATS

SYNOPSIS

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Introduction

Alcohol is amongst the most broadly utilized and abused drugs adding to an assortment of restorative and financial issues. Alcohol is a psychoactive medication—its main impact on the cerebrum is as a focal sensory system depressant [1]. Ceaseless extreme intake of ethanol for extensive periods causes tolerance and reliance on Ethanol. Besides, harsh savoring results in organ and tissue harm. Alcohol is legal and socially adequate in many nations and is additionally the most utilized drug around the world [2]. It is consumed predominantly for relaxation, fun and social reasons. Despite the fact that a larger part of individuals consumes alcohol in moderate amounts which can really prompt positive health results, the worldwide burden of illness identified with alcohol consumption is higher [3]. It is for the most part in light of the fact that incautious alcohol consumption isn't connected just with long haul health impacts but instead with intense results, for example, vehicle crashes, drowning or freezing. Such antagonistic results lead to unexpected deaths and inability, particularly among youngsters. Overabundance drinking is likewise connected with diminished profitability, crime, brutality or identity crumbling. Alcohol abuse can prompt alcohol dependence which is a sickness that may include craving, loss of control, tolerance and physical dependence [4]. Chronic consumption of alcohol prompts numerous neuroadaptations and makes an allosteric state. Modification in brain reward and stress framework occurs because of such changes [5]. These impacts feature themselves through neuroadaptations in nucleus accumbens, hippocampus, amygdala, prefrontal cortex, and numerous different regions. Neurotransmitter frameworks like dopamine, gamma-aminobutyric acid (GABA), glutamate, serotonin, and a few neuropeptides referred to progress toward becoming deregulated because of ceaseless drinking and alcohol misuse [6].

The alcohol withdrawal syndrome is a state that shows up after decrease or sudden end of consistent heavy drinking in individuals experiencing alcohol dependence [7]. According to the World Health Organization (WHO) report 76.3 million individuals over the world have alcohol use disorders and reliance which results in 1.8 million deaths for each year. Alcohol dependence is characterized by enthusiastic alcohol seeking, loss of control in restricting alcohol intake, and appearance of a withdrawal syndrome without alcohol [8-9]. Abstinence from chronic alcohol consumption prompts over-excitation of glutamatergic neuronal synaptic transmission in the amygdala, which results in anxiety-like behavior which is characterized by a raised negative emotional response. Negative enthusiastic reactions originating from ethanol withdrawal side

effects are the detailed reasons that lead to backsliding to alcohol use. Withdrawal symptoms generally show up inside 6-24 h of restraint from alcohol [10]. Manifestations incorporate a rise in blood pressure, anxiety, agitation, hyperreflexia, tremors, queasiness or heaving, sleep deprivation, perspiring, tachycardia, tremors, fractiousness and depression, which may progress to severe conditions, for example, seizures, delirium tremens (DTs), confusion, excessive hallucinations and extreme autonomic instability. Manifestations may prompt serious forms marked by seizures, heart failure and death happen in 5% to 10% of patients [1,11,12]. One of the factors in human drunkards which lead them to heavy drinking is the utilization of alcohol to get calmed of the withdrawal manifestations or to re-experience a similar dimension of the compensating impacts recently experienced with ethanol [13]. Alcohol withdrawal works as an unconditioned stressor for invigorating unconditioned withdrawal reactions, which prompts the actuation of a few cerebrum areas, particularly the regions that are engaged with the tweak and articulation of anxiety and depressive-like behaviors, for example, the amygdala, prefrontal cortex, hypothalamus, and hippocampus. Among the different limbic structures, the amygdala and hypothalamus enactment are believed to have an imperative role in modulating ethanol withdrawal anxiety-like behaviors [14,15]. Ethanol utilization prompts neurobiological and behavioral alterations which are intervened by NMDA (N-Methyl-D-aspartic acid) excitatory and $GABA_A$ (γ-aminobutyric acid) inhibitory receptor frameworks. Ethanol reliance results in a diminished GABA-A neuroreceptor response and up-regulation in excitatory NMDA receptors, this regulate ethanol drinking reinforcement reward, resilience, dependence, and withdrawal. GABAergic and NMDA/glutamatergic frameworks can be the imperative medication focuses on accomplishing a long haul forbearance from alcohol [1-2]. At present treatment choices for alcohol withdrawal syndrome are not many, for example, Benzodiazepines, Disulfiram, Naltrexone, Acamprosate and among all benzodiazepines are the main medication of choice for treatment of alcohol withdrawal syndrome, yet their utilization is related to different symptoms, for example, sedation, psychological hindrance and have addictive properties which make a constraint to their use. Subsequently, the assurance of new powerful and therapeutically beneficial medications for the treatment of alcohol withdrawal indications is imperative [16,17].

Plants are the critical sources of medication and about countless drugs (25%– 30%) being used are derived from plants. Viability of restorative plants in the management of diseases is apparent, and the World Health Organization has perceived its utilization in essential medicinal services

conveyance framework [18]. Herbal drugs are perceived as the significant source of therapeutics and are the principal choices in medicinal services treatment these days as they are better compatible with the human body and have insignificant reactions and desirable results. In view of a blend of conventional utilization and continuous scientific research, the capability of plant-based preparations for the aversion of sicknesses and improvement in wellbeing quality has pulled in much research consideration [19]. Polyherbal preparations have accomplished wide acknowledgment in contrast with unrefined plant extracts and are broadly utilized for treating different disorders because of convenience, decrease in dose proportion and simplicity of administration. Polyherbal preparations are well known worldwide as restorative operators, in different ailments that sway the quality of life. A large portion of the synthetic medications give symptomatic alleviation by following up on a solitary molecular target; the multitarget reactions of Polyherbal preparations are turned out to be helpful in interminable conditions, for example, cancer, diabetes, osteoporosis, etc. It ought to be noticed that herbal preparations have been esteemed for their additional viability because of the synergistic impact of numerous herbs [20-22].

Hypericum perforatum usually known as St. John's wort, family Hypericaceae is accounted for to have anti-anxiety, antidepressant, antidiabetic, pain relieving and other mood disorders related properties. The active phytoconstituents present in *Hypericum perforatum* are hypericin, hyperforin, isohypericin, pseudohypericin, quercetin, luteolin, rutin, kaempferol, and hyperoside [23]. *Bacopa monnieri* usually known as Brahmi family Scrophulariaceae is accounted for to have defensive impacts against neurological disorders like epilepsy, depression, insanity, stress, psychosis, and sleep deprivation. Phytoconstituents present in *Bacopa monnieri* are brahmine, bacoside A, bacoside B, bacopasaponins A, B and C, herpestine and D-mannitol [24]. *Centella asiatica* commonly known as Gotu kola family Apiaceae is accounted for to have different pharmacological activities like an antiepileptic, antidepressant, nervine tonic, rejuvenant, sedative and tranquilizer. *Centella asiatica* contains phytoconstituents like asiaticosides, madecassoside, Asiatic acid, pentacyclic triterpenoid saponins, madasiatic acid, campesterol, and sitosterol [25]. *Withania somnifera* usually known as Ashwagandha family Solanaceae is professed to have antianxiety, pain relieving, antistress and anti-inflammatory properties. *Withania somnifera* contains phytoconstituents like withaferins, withanolides, withananine, sitoindoside, isopellertierine, somniferine, somnine and tropine [26]. *Oscimum sanctum*

commonly known as Tulsi family Lamiaceae have pain relieving, antistress, antipyretic, anticonvulsant, neuroprotective, immunomodulatory, calming, and memory enhancer properties. *Oscimum sanctum* has active phytoconstituents like euginal, eugenol, carvacrol, urosolic corrosive, rosmarinic corrosive, apigenin and caryophyllene [27]. *Camellia sinensis* commonly known as Green tea family Theaceae have pharmacological properties like anti-Parkinson, anti-aging, antistroke and anti-Alzheimer's. The active phytoconstituents present in *Camellia sinensis* are gallocatechin, catechin, gallocatechingallate, quercetin, catechingallate, myricetin, and kaempferol [28].

These plants have different pharmacological properties and have been utilized for the preparation of polyherbal preparations in the Ayurvedic system of drugs for treating many disorders. In past investigations, it has been discovered that a blend of *Hypericum perforatum, Bacopa monnieri*, and *Camellia sinensis* have synergistic cell reinforcement properties. Keeping in view the diverse pharmacological properties and synergistic activities of these medicinal plants a Polyherbal preparation POL-6 containing *Withania somnifera, Camellia sinesis, Hypericum perforatum, Centella asiatica, Bacopa monniera* and *Ocimum sanctum,* and plant extracts was developed, standardized and evaluated for the alcohol deprivation effects following long term voluntary alcohol consumption in rats.

Objectives

1. To prepare and standardize the POL-6 using Indian Pharmacopoeia standards.
2. To calculate the LD_{50} of the POL-6 using OECD guidelines of acute and sub-acute toxicity in rats.
3. To screen the POL-6 in ethanol abstinence-induced behaviors like anxiety, depression and seizures in rats and to carryout comparative mRNA expression studies in control and ethanol abstinence animals for $GABA_A$ receptor and NMDA-glutamate receptor subunits.

Objective 1: To prepare and standardize the POL-6 using Indian Pharmacopoeia standards.

Materials and Methods

Preparation of POL-6

The dried roots of *Withania somnifera* (Bath number: EBD–18), dried whole plant of *Centella asiatica* (Bath number: ERD–040), *Bacopa monnieri* (Bath number: ERD–92) and dried leaves of *Ocimum sanctum* (Bath number: RHD 283), *Camellia sinensis* (Bath number: ERM–23) has been purchased from Natural Remedies, Bangalore. The aerial parts of *Hypericum perforatum* L. have been collected from the herbal garden of the Jaypee University, Solan, Himachal Pradesh (H. P.) and authenticated by Dr. Y. S. Parmar University of Horticulture and Forestry, Solan, H. P. A voucher specimen of the said plant has been deposited to UHF–Herbarium (Field book number: 13420). The individual plants were coarsely powdered with a mechanical grinder and passed through sieve number 60 and stored in an airtight container. The coarsely powdered individual plants were defatted with petroleum ether (30–40 °C) and the defatted air dried individual plant powder was further subjected to hydroalcoholic extraction (70% v/v) using soxhlet apparatus at 50 °C for 48 h. The obtained solvent was recovered using a rotary evaporator (Heidolph, Germany) and the semisolid mass has been further lyophilized (New Brunswick) to obtained dry powder. The obtained dry powder of individual plant was stored at 2–8 °C till further use. For the preparation of POL–6, most effective doses of the plants were selected from the literature and the powdered extract of each plant (*Bacopa monnieri, Hypericum perforatum, Centella asiatica, Withania somnifera, Camellia sinesis and Ocimum sanctum)* was weighed separately and mixed in (2:1:2:2:1:2) proportions using a double cone blender. The mixture was passed through sieve number 40 to obtain a homogeneous blend and was packed in a tightly closed container. The obtained mixer was then stored in a cool and dry place until further use.

Standardization of POL-6

Sensory characteristics of POL-6 like colour, odour, shape, size, taste, texture and fracture were identified using organoleptic evaluation.

Physiochemical investigation of POL-6 [29]

Loss on drying

Loss on dying is the loss of mass expressed as percent w/w. 10g of samples of POL-6 were accurately weighed in a dried and flat weighing bottle and dried at 105°C for 5hrs. Percentage was calculated with reference to initial weight.

Determination of total ash

A high ash value is indicative of contamination, adulteration substitution or carelessness in preparing the formulation. Ashing involves an oxidation of the components of the products. For its determination, 2g powdered material of POL-6 was placed in a suitable tared crucible of silica previously ignited and weighed. The powder was spread into an even layer and weighed accurately. The material was incinerated by gradually increasing the heat, not exceeding 450°C until free from carbon, cooled in desiccators, weighed and percentage ash was calculated by taking in account the difference of empty weight of crucible & that of crucible with total ash.

Determination of Acid insoluble ash

The ash obtained as above was boiled for 5min with 25ml of dilute hydrochloric acid; the insoluble matter was hot water and collected on an ash less filter paper, washed with ignited to constant weight. The percentage of acid-insoluble ash with reference to the air-dried drug was calculated.

Determination of solvent extractive values of POL-6 [30]

Alcohol soluble extractive value

5g of coarsely powdered air-dried POL-6 was macerated with 100 ml of alcohol in a closed flask for twenty-four hours, shaking frequently during six hours and allowing standing for eighteen hours. It was then filtered rapidly; taking precautions against loss of solvent. 25ml of the filtrate was evaporated to dryness in a tared flat-bottomed shallow dish at 105°C to constant weight and weighed. The percentage of alcohol-soluble extractive was calculated with reference to the air dried drug and is represented as % value.

Water soluble extractive value

5g of coarsely powdered air-dried POL-6 was macerated with 100 ml of chloroform water in a closed flask for twenty-four hours, shaking frequently during six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent. 25ml of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish at 105°C to constant weight and weighed. The percentage of water-soluble extractive was calculated with reference to the air-dried drug and is represented as % value.

Determination of pH

1% solution of POL-6 was prepared in distilled water and pH was determined using pH meter

Physical characteristics of POL-6 powder [29]

Bulk density and Tape density

The term bulk density refers to a measure used to describe a packing of particles or granules. The equation for determining bulk density (Db) is: Db = M/Vb, Where M is the mass of the particles and Vb is the total volume of the packing. The volume of the packing can be determined in an apparatus consisting of a graduated cylinder mounted on a mechanical tapping device (Jolting Volumeter) that has a specially cut rotating can. 100gm of weighed POL-6 powder was taken and carefully added to the cylinder with the aid of a funnel. Typically, the initial volume was noted and the sample was then tapped until no further reduction in volume was noted. The initial volume gave the Bulk density value and after tapping the volume reduced, giving the value of tapped density.

Angle of repose

In general, powders with angle of repose greater than 50 degree have unsatisfactory flow properties, whereas minimal angle close to 25 degrees correspond to very good flow properties. The fixed funnel and the free standing cone method employs a funnel that is secured with its tip at a given height, which was taken 2.5 cm (H), above the graph paper that is place on flat horizontal surface. Powder was carefully poured through the funnel until the apex of the conical pile just touched the tip of the funnel. Tan $\alpha = H/R$ or $\alpha = \arctan H/R$

Where α is the angle of repose, R being the radius of the conical pile.

Hausner's ratio

Hausner's ratio is related to inter particle friction and as such can be used to predict the powder flow properties. Powders with low inter particle friction such as coarse spheres; have a ratio of approximately 1.2, whereas more cohesive, less flowable powders such as flakes have a Hausner's ratio greater than 1.6. The equation for measuring the Hausner's ratio is:

Hausner's ratio = Df / Do, where Df = Tapped density and Do = Bulk density.

Carr's index

Carr's index is a method of measuring the powder flow from bulk density. The equation for measuring Carr's index is: % compressibility = (Df-Do/Do) × 100 where Df = Tapped density and Do = Bulk density.

Phytochemical screening of POL-6 [31]

Pol-6 was subjected to phytochemical screening tests to evaluate the presence of chemicals constituents like alkaloids, flavonoids, proteins, glycosides, fixed oils, phenolics, sugars and tannins.

Total flavonoid content quantification

Total flavonoid content in POL-6 was calculated by aluminum chloride assay method and rutin was used as a reference compound. A volume of 0.5 ml POL-6, distilled water (2ml) and 0.15 ml of sodium nitrite (5% w/v) was made. Add 0.15 ml of aluminum trichloride (10%) after 5 min, and after the incubation of 6 min, To a reaction mixture, 2ml of NaOH (4% w/v) was added. 5ml of total volume was made with distilled water and incubated for more 15 min. When the pink color is observed in the above mixture, absorbance was measured against blank at 510 nm. The total flavonoid content was represented in mg of rutin equivalent per gram of POL-6 [31].

Total phenolic content quantification

The determination of phenolic content in POL-6 was done using Folin and Ciocalteu method was used for calculating the. Folin and Ciocalteu phenol reagent was mixed with 100µl of test in 1:10 proportion. The sample mixture was incubated for 1 min at room temperature followed by addition of 1.5 ml of 20% sodium carbonate. The reaction mixture (10 ml) was incubated at room temperature for 90 min (in dark). The sample absorbance was read at 725nm and phenolic content was calculated on the basis of Gallic acid equivalents, GAE/g of dry plant material.

Quantification of condensed tannin content

400µL of POL-6 was added to hydrochloric acid concentrated (1.5 ml) and 4% solution of vanillin (3 ml) in methanol. The mixture was incubated for 15 min and the absorbance was taken at 500 nm. The tannin content was expressed as mgCE/g of test, utilizing a Catechin calibration curve.

Qualitative phytochemical profiling of POL-6 by HPLC

POL-6 (5mg) was dissolved in 10mL methanol (80%) and 25ppm solution was made by diluting it further. The solution was filtered with 0.22 micron syringe filter. The HPLC system (Agilent

Technologies) consists of an LC-binary pump, diode array detector, EZ-chrom system controller, and Innoval C18 (4.6×250mm) column. For separation, 0.14 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) was added to 900ml of HPLC grade water and orthophosphoric acid (0.5ml) was dissolved in above mixture. The volume of the mixture was made upto1000 ml with water and then filtered through membrane filter (0.45 µ). After filtration the solution was placed in a sonicator for 3 minutes. The prepared solution was considered as the mobile phase gradient solvent (A) and the Acetonitrile was used as the solvent (B). 20µl sample was injected through the SIL-HTC Shimadzu Autosampler. The conditions for solvent system were 20:80 ratios, flow rate 1.5ml/min and a run time of approximately 45 minutes. The chromatogram was obtained at wavelength of 227nm.

Liquid Chromatography- mass spectroscopy profiling of POL-6

The major chemical constituents present in the POL-6 were identified by using liquid chromatography-mass spectroscopy (LC-MS). Phenomenex C18 (150 × 4 mm i.d., 5 µ) with single quadrupole mass spectrometry analyzer was used for the liquid chromatography separation. 0.5% formic acid–acetonitrile (75:25%) was used as the mobile phase. The flow rate was 0.5 ml/min. The solvent was controlled by isocratic elution. The column temperature was kept at 30°C. The MS spectra were gained in the positive ion mode. Nebulizing pressure of the drying gas (N_2) was of 25 psi, temperature was 350^{o}C at a gas flow rate of 6 ml/min. About 0.5 g of POL-6 was diluted with methanol and filtered with 0.22 µm nylon filter prior to examination. A 5 µl volume of the POL-6 was injected onto the column for examination. The mass fragmentations were identified by using spectrum database for organic compounds.

Quantification of major constituents present in POL-6 by HPTLC

The major constituents like Withaferin A, Quercetin, β-sitosterol, Catechin, Caffeic acid and Rutin were quantified in the POL-6 using a HPTLC instrument with CAMAG Linomat V automatic sample applicator, TLC scanner III, Camag twin trough chamber 10×10 cm, and WinCATS software in present study. POL-6 (suspended in methanol) and standard solutions (each 5 µl) (suspended in methanol) were applied in the form of a band having bandwidth 8 mm; distance between the bands 14 mm and a constant application rate of 150 nL s^{-1} using a microsyringe (Hamilton-Bonaduz Schweiz, Linomat syringe, 500 µl size) to a silica gel precoated 60 F254 TLC plates (10 × 10 cm with 200 µm thickness). TLC plates were then placed

under the different mobile phases (Withaferin A; chloroform: methanol,:0.5, v/v, Quercetin; Toluene: methanol, 7:3, v/v, β-sitosterol; n-hexane: ethyl acetate, 8:2, v/v, Catechin, Caffeic acid; toluene: ethyl acetate: formic acid, 5:4:1, v/v/v, Rutin; ethyl acetate: formic acid: acetic acid: water, 10:1.1:1.1:2.6, v/v/v/v) in a glass developing chamber and development was performed in ascending manner to a distance of 8 cm. After the development, the densitometric scanning of the air-dried plate was performed by the help of TLC scanner operated in reflectance-absorbance mode, slit dimensions: 6×0.45 at 254 nm. The calibration curve of all the standards was drawn. The sample and standard spots were applied on TLC plates and the contents of metabolites were analyzed using regression equation from the calibration plot and expressed as % w/w.

Evaluation of antioxidant activity of POL-6

Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

POL-6 was assayed for the DPPH scavenging activity as per its ability to bleach the stable DPPH radicals. Different concentrations 5, 10, 20, 40, 80, 100µg/ml of POL-6 and standard (ascorbic acid) were prepared. 500µl of each concentration was mixed with 500µl of DPPH. In the control test tube, 500µl of DMSO and 500µl of DPPH was mixed which will serve as an absorbance control. The absorbance (Abs) was taken at 517nm after incubating all the samples at 37^{o}C for 30 minutes in the dark. The equation (Scavenging DPPH (%) = [(control Abs –sample Abs)/control Abs] × 100) was used for calculating the percentage inhibition of the samples. Further IC_{50} was calculated from the percentage inhibition which shows the level at which 50% of the free radicals was scavenged by the test sample.

Nitric oxide scavenging assay

Nitric oxide when reacts with oxygen produces nitrate and nitrite; the quantities of these stable products can be determined by using Griess reagent method. At various concentrations (5, 10, 20, 40, 80 and 100 µg/ml) POL-6 and ascorbic acid standard were prepared. This was followed by incubation period at 37°C for 5 hrs with the addition of 5mM of sodium nitroprusside solution in 0.025 M phosphate buffer (pH-7.4). A proportionate measure of methanol was served as a control. After incubation 500µl of incubated solution was diluted with 500µl of Griess reagent and absorbance was measured at 546 nm. The percentage scavenging or antioxidant by test

samples at different concentrations was analyzed using equation (Scavenging NO (%) = [(Abs control –Abs sample)/Abs control] × 100) further IC_{50} value was calculated.

Result and Discussion

POL-6 preparation

Air dried powdered extract of plants (*Bacopa monnieri, Hypericum perforatum, Centella asiatica, Withania somnifera, Camellia sinesis and Ocimum sanctum*) was weighed independently and mixed in 2:1:2:2:1:2 proportions respectively utilizing a twofold cone blender. Weight of each plant extract used for POL-6 preparation is depicted in the Table 1.

Table 1: Ratios of the plant extracts used for the preparation of POL-6.

Sr. No.	Plant	Ratio	Weight in grams for 100 g
1.	*Bacopa monnieri*	2	20 g
2.	*Hypericum perforatum*	1	10 g
3.	*Centella asiatica*	2	20 g
4.	*Withania somnifera*	2	20 g
5.	*Camellia sinensis*	1	10 g
6.	*Ocimum sanctum*	2	20 g

Organoleptic evaluation of POL-6

As part of standardization procedure, the prepared POL-6 was tested for relevant physical and chemical parameters. Organoleptic evaluation revealed that POL-6 is a powder with dark brown color, pungent odor, bitter taste and fine texture.

Physiochemical investigation and solvent extractive values of Pol-6

Quality tests on POL-6 were performed for Loss on Drying at 105ºC (4.12 ± 0.121), Water soluble extractive value (%) (46.138 ± 0.213), Alcoholic soluble extractive value (%) (69.48 ± 0.485), Total ash (%) (15.30 ± 0.023), Acid insoluble ash (%) (0.62 ± 0.012), pH (1% aq. Soln.)

(7.14 ± 0.217) and were found to be within standard ranges. The results are expressed as mean (n=3) ± Standard deviation (SD) and shown in Table 2.

Table 2: Physiochemical characteristics of POL-6

S.no.	Parameters	Percentage Mean (N=3) ± SD
1	Loss on Drying 105ºC	4.12 ± 0.121
2	Water soluble Extractive Value (%)	46.14 ± 0.213
3	Alcoholic Soluble Extractive Value (%)	69.48 ± 0.485
4	Total Ash (%)	15.30 ± 0.023
5	Acid Insoluble Ash (%)	0.62 ± 0.012
6	pH	7.14 ± 0.217

The results are expressed as mean (n=3) ± Standard deviation (SD)

Physical characteristics of POL-6

Physical properties of the Pol-6 like Bulk density, Tap density, Carr's compressibility index, Haussner's ratio, Angle of repose were determined and results were tabulated in Table 3.

Table 3: Physical characteristics of POL-6

S. no.	Parameters	Results
1	Bulk Density	0.441 ± 0.001 (gm/ml)
2	Tapped Density	0.612 ± 0.011 (gm/ml)
3	Carr's Index	38.77 %
4	Hausner's Ratio	1.38 ± 0.2
5	Angle of Repose	27.2 ± 0.3

Phytochemical screening of POL-6

The qualitative phytochemical analysis of POL-6 revealed the presence of flavonoids, alkaloids, carbohydrate, glycosides, steroids, tannin and phenolics (terpenoids). The observations made during phytochemical analysis are shown in Table 4.

Table 4: Phytochemical screening of POL-6

Compound	Detection Method	Inference
Flavonoids	Shinoda test	+
	Zinc hydrochloride test	+
Alkaloids	Dragendroff's reagent	+
	Hager's reagent	+
	Mayer's reagent	+
	Wagner's reagent	+
Phenolic and Tannins	Ferric Chloride Test	+
	Vanillin hydrochloride test	+
Steroids & triterpenoids	Salkowski Test	+
	Libermann-Burchard test	+
Glycosides	Fehling's test	+
Saponin glycosides	Froth test	+
Coumarin glycosides	Coumarin Glycoside	+
Anthraquinone glycosides	Borntrager's test	+

Cardiac Glycosides	Keller Killian test	+
Amino acids	Millon's test Ninhydrin test	+ +
Carbohydrates	Molisch's test	+
Pentoses	Barfoed Test	+

Presence (+), Absence (-)

Quantification of total phenolic, flavonoid and tannin content in POL-6

Phytoconstituents confirmed by qualitative phytochemical screening were further quantified by quantitative phytochemical analysis. Total phenolic content of POL-6 was calculated as mg of Gallic acid (GAE) equivalents/g dry weight (DW) (mg GAE/g DW) and was found to be 316.66±0.28 mg GAE/g. The total flavonoid content of POL-6 was calculated as mg of quercetin (QR) equivalents/g dry weight (mg QR/g DW) and was found to be 481.14±0.34 mg QR/g. Tannin content of POL-6 was calculated as mg of catechin (CE) equivalents/g dry weight (mg CE/g DW) and was found to be 100.43±0.41. The results of quantitative phytochemical screening are depicted in the Table 5.

Table 5: Quantitative analysis of phenolic, flavonoid and tannin content in Pol-6.

Polyphenol content (mg of GAE/g DW)	**Flavonoid content (mg of QR/g DW)**	**Tannin content (mg of CE/g DW)**
316.66±0.28	481.14±0.34	100.43±0.41

Qualitative phytochemical profiling of POL-6 by HPLC

Qualitative phytochemical of POL-6 was also performed by HPLC analysis. HPLC chromatogram showed eleven peaks indicating the presence of eleven major compounds. Different peaks, retention time, peak area and area percentage is shown in Fig 1, Table 6. The identification and quantifications of the compounds was further studied through LC-MS and HPTLC.

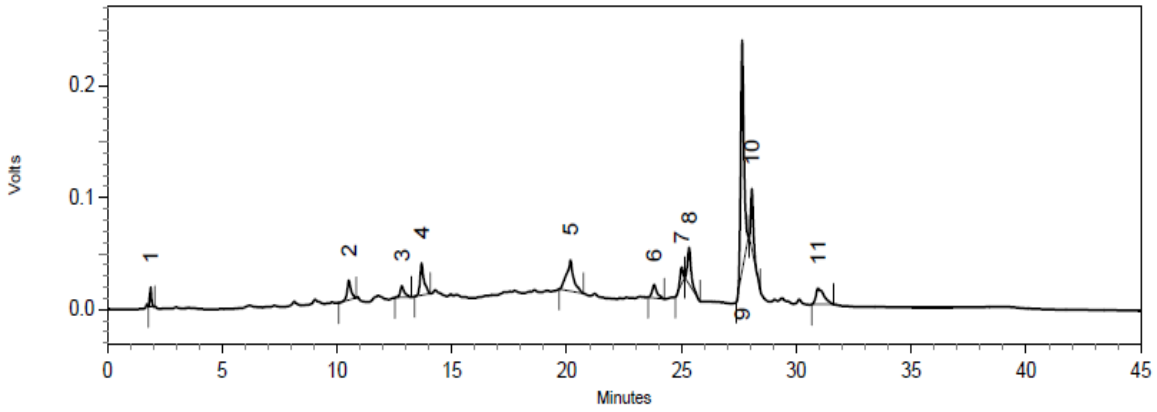


Fig 1: HPLC Chromatogram of POL-6.

Table 6: HPLC retention time and peak area indicating the presence of 11 major compounds.

Sr. No.	Retention Time	Area	Area Percent
1.	1.892	90417	1.90
2.	10.517	201504	4.23
3.	12.825	136234	2.86
4.	13.683	343799	7.22
5.	20.167	593571	12.46
6.	23.808	171106	3.59
7.	25.008	118638	2.49
8.	25.325	352297	7.39
9.	27.642	1990010	41.77
10.	28.058	417285	8.76
11.	30.942	349156	7.33

Liquid chromatography-mass spectroscopy profiling of POL-6

Mass calculated for $C_{28}H_{38}O_6$ (Withaferin A) exact mass 470.606 g/mol, fragmentation pattern ((steroid –OH) + H^+), found mass 287.0355; $C_{28}H_{38}O_6$ (Withanolide) exact mass 470.606 g/mol, fragmentation pattern ((steroid –OH) + H^+), found mass 287.0355; $C_{15}H_{10}O_6$ (Luteolin) exact mass 286.24 g/mol, fragmentation pattern (+ H^+), found mass 287.0355; $C_{29}H_{50}O$ (β-Setosterol) exact mass 414.71 g/mol, fragmentation pattern (Isopropyl- CH_3), found mass 287.0355; $C_{15}H_{10}O_7$ (Quercetin) exact mass 302.236 g/mol, fragmentation pattern (+ Na^+), found mass 325.0721; $C_{41}H_{68}O_{13}$, (Bacoside A) exact mass 769 g/mol, fragmentation pattern (-sugar + Na^+), found mass 463.0686; $C_{30}H_{16}O_8$ (Hypericin) exact mass 504.44 g/mol, fragmentation pattern (-2Ketone,-OH, -Anthracene one connection), found mass 464.01; $C_{27}H_{30}O_{16}$ (Rutin) exact mass 610.52 g/mol, fragmentation pattern (+H^+), found mass 611.1354; $C_{10}H_{18}O$ (Linalool) exact mass 154.25 g/mol, fragmentation pattern (-CH_2), found mass 142.11; $C_9H_8O_4$ (Caffeic acid) exact mass 180.16 g/mol, fragmentation pattern (+H^+), found mass 182.9503; $C_{15}H_{14}O_6$ (Catechin) exact mass 290.26 g/mol, fragmentation pattern (-O-hydroxyphenol), found mass 182.9503; $C_{10}H_{12}O_2$ (Eugenol) exact mass 164.2 g/mol, fragmentation pattern (-H^+), found mass 163.0318; $C_6H_{14}O_6$ (D-Mannitol) exact mass 182.172 g/mol, fragmentation pattern (+H^+), found mass 184.1210; $C_{28}H_{38}O_6$ (Withanone) exact mass 470.6 g/mol, fragmentation pattern (-Pyran, -CH_3), found mass 327.22 Fig 2.

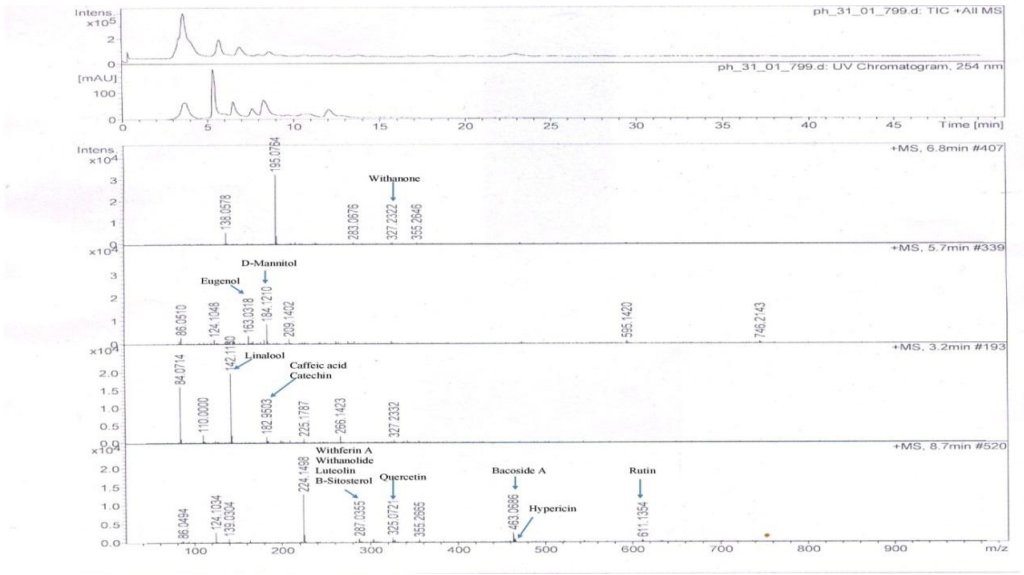


Fig 2: LC-MS spectrum of POL-6 showing peaks and molecular mass of different compounds.

Quantification of major constituents present in POL-6 by HPTLC

Major Phytoconstituents identified by LC-MS was further quantified by the HPTLC. Quantification of six compounds Withaferin, Quercetin, Caffeic acid, β-Sitosterol, Rutin and Catechin was performed by HPTLC.

Quantification of Withaferin A in POL-6 by HPTLC

Mobile phase consisting chloroform: methanol (9.5:0.5, v/v) showed sharp peaks with Rf value of 0.59 for Withaferin A (Fig 3A). Withferin A found in the POL-6 was 0.921% w/w of POL-6. HPTLC chromatogram of POL-6 and Withaferin A is presented in the Fig 3 B.

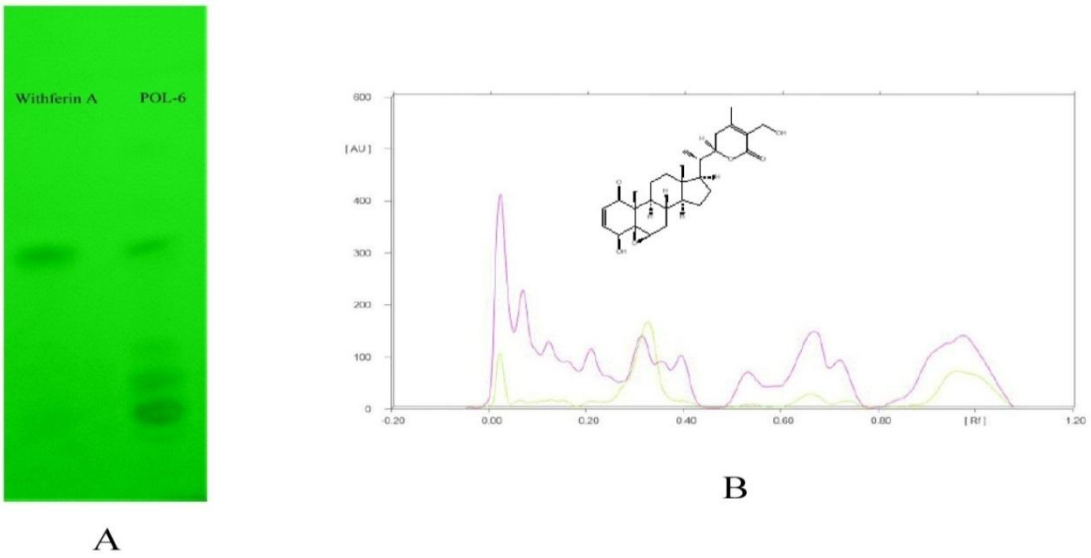
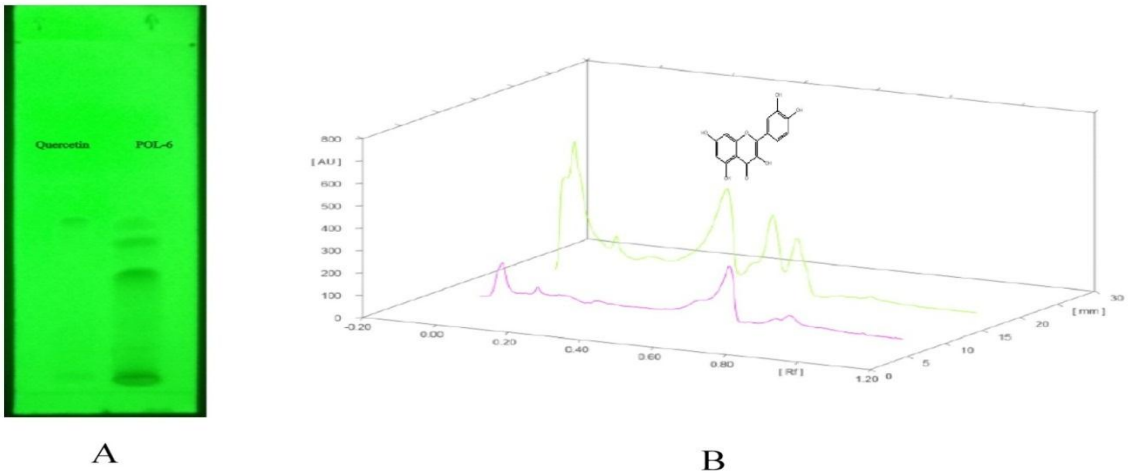


Figure 3 TLC and HPTLC estimation of Withaferin A in POL-6. (A) TLC plate of POL-6 with Withaferin A (B) HPTLC chromatogram of POL-6 and Withaferin A.

Quantification of Quercetin in POL-6 by HPTLC

Mobile phase consisting Toluene: methanol (7:3, v/v) showed sharp peaks with Rf value of 0.65 for Quercetin (Fig 4 A). Quercetin found in the POL-6 was 1.50% w/w of POL-6. HPTLC chromatogram of POL-6 and quercetin is presented in the Fig 4 B.

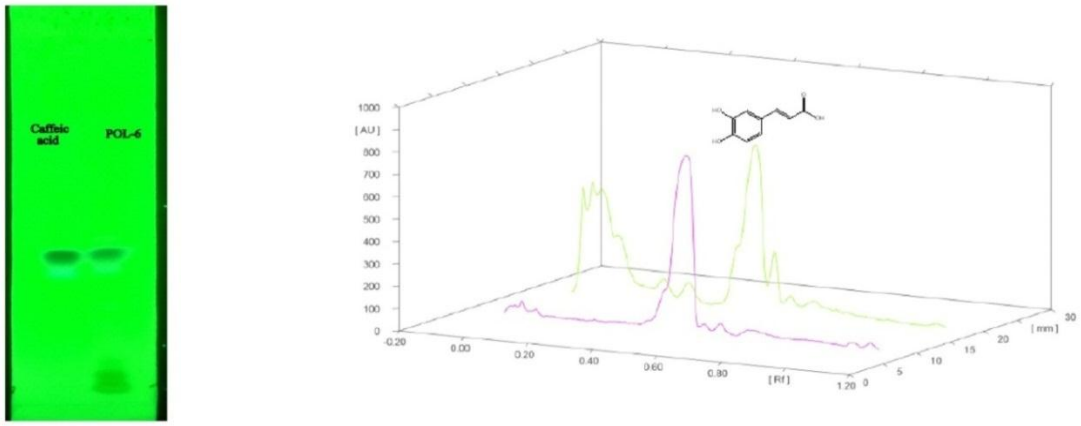


A B

Figure 4: TLC and HPTLC estimation of quercetin in POL-6. (A) TLC plate of POL-6 with Quercetin (B) HPTLC chromatogram of POL-6 and quercetin.

Quantification of Caffeic acid in POL-6 by HPTLC

Mobile phase consisting toluene: ethyl acetate: formic acid (5:4:1, v/v/v) revealed sharp peaks with Rf value of 0.49 for Caffeic acid (Fig 5 A). Caffeic acid found in the POL-6 was 1.059% w/w of POL-6. HPTLC chromatogram of POL-6 and Caffeic acid is presented in the Fig 5 B.



A B

Figure 5: TLC and HPTLC estimation of Caffeic acid in POL-6. (A) TLC plate of POL-6 with Caffeic acid (B) HPTLC chromatogram of POL-6 and Caffeic acid.

Quantification of Rutin in POL-6 by HPTLC

Mobile phase consisting ethyl acetate: formic acid: acetic acid: water (10:1.1:1.1:2.6, v/v/v/v) showed sharp peaks with Rf value of 0.86 for Rutin (Fig 6 A). Rutin found in the POL-6 was 0.86% w/w of POL-6. HPTLC chromatogram of POL-6 and Rutin is presented in the Fig 6 B.

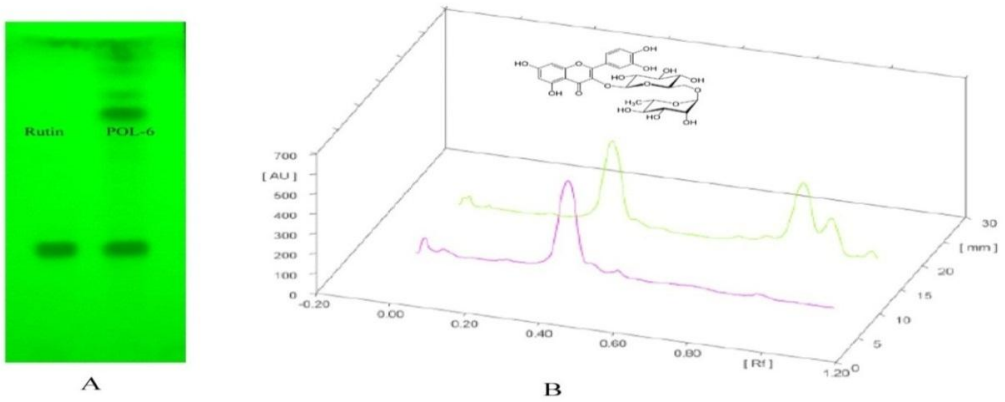


Figure 6 : TLC and HPTLC estimation of Rutin in POL-6. (A) TLC plate of POL-6 with Rutin (B) HPTLC chromatogram of POL-6 and Rutin.

Quantification of β-Sitosterol in POL-6 by HPTLC

Mobile phase consisting n-hexane: ethyl acetate (8:2, v/v) showed sharp peaks with Rf value of 0.61 for β-Sitosterol (Fig 7 A). β-Sitosterol found in the POL-6 was 0.60% w/w of POL-6. HPTLC chromatogram of POL-6 and β-Sitosterol is presented in the Fig 7 B.

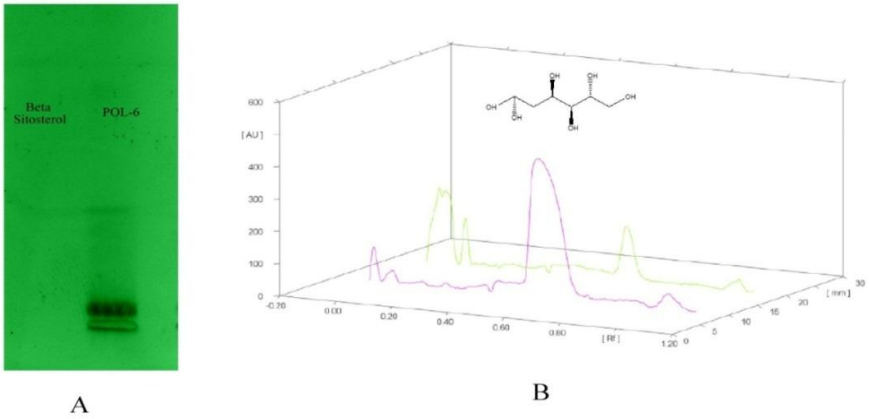


Figure 7: TLC and HPTLC estimation of β-Sitosterol in POL-6. (A) TLC plate of POL-6 with β-Sitosterol (B) HPTLC chromatogram of POL-6 and β-Sitosterol.

Quantification of Catechin in POL-6 by HPTLC

Mobile phase consisting toluene: ethyl acetate: formic acid (5:4:1, v/v/v) revealed sharp peaks with Rf value of 0.22 for Catechin (Fig 8A). Catechin found in the POL-6 was 2.86% w/w of POL-6. HPTLC chromatogram of POL-6 and Catechin is presented in the Fig 8 B.

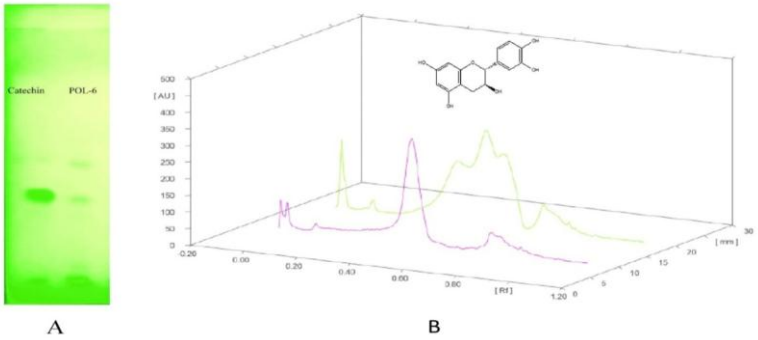


Figure 8: TLC and HPTLC estimation of Catechin in POL-6. (A) TLC plate of POL-6 with Catechin (B) HPTLC chromatogram of POL-6 and Catechin.

DPPH radical scavenging assay

Free radical (DPPH) scavenging activity of POL-6 was compared with standard ascorbic acid. The IC_{50} values were determined from the percentage inhibitory concentrations and were found to be POL-6 (22.54±0.21 µg/ml) and ascorbic acid (18±0.18 µg/ml). The obtained results are depicted in Table 7.

Nitric oxide radical scavenging activity

The free radical scavenging activity of POL-6 against nitric oxide formation was compared with ascorbic acid (standard). The IC_{50} values were calculated from the percentage inhibitory concentrations and found to be POL-6 (21.38±0.17 µg/ml) and Ascorbic acid (17.99 ± 0.17 µg/ml). The obtained results are depicted in Table 7.

Table 7: Free radical scavenging activity of POL-6

Sample Name	**$IC_{50/DPPH}$ (µg/ml)**	**$IC_{50/NO2}$ (µg/ml)**
POL-6	22.54±0.21	21.38±0.13
Ascorbic acid	18 ± 0.18	17.99±0.17

Objective 2: To calculate the LD_{50} of the POL-6 using OECD guidelines of acute and sub-acute toxicity in rats.

Animals and housing

Wistar rats male and female (8–12 weeks, weighing 170–220 g) were separately housed in groups of five animals per cage under controlled temperature (25 ± 2°C), light and dark cycle and food and water *ad libitum* in the animal house of the Central Animal Facility, Jaypee University, Solan, H.P., India. All experiments were carried out after approval and under strict compliance as per the guidelines of the Institutional Animal Ethics Committee (3/GLG/2014/JUIT/IAEC), constituted as per CPCSEA (1716/PO/a/13/CPCSEA).

Acute toxicity study

Five healthy female Wistar rats (non-pregnant and nulliparous) were used for the study according to the Organization for Economic Cooperation and Development (OECD-2008) test guidelines 425. Initially, a single dose of POL-6 (2000 mg/kg) was given orally to one female rat under prior fasting conditions for 8 hours. Later on, after 48 hours the same dose was given to the remaining four female rats. Alongside a negative control group, vehicle treatment (1% sodium carboxymethyl cellulose) was given to another five female rats. Treated animals were observed for general activity (conscious state), muscle coordination, corneal reflexes, tremors, convulsions, straub, sedation, anesthesia, lacrimation, piloerection, ptosis, salivation, and mortality regularly for 30 min and then periodically for 4 and 24 h after POL-6 administration and observed daily thereafter for 14 days. The body weight, water intake and feed consumption by the animals were also recorded [32].

Sub-acute toxicity study

The sub-acute safety assessment was conducted as per the OECD guidelines–407 for repeated 28 days of daily dosing. A total of 52 male and female Wistar rats were divided into six groups. Each three groups G_2, G_3, G_4 consisted of five males and five females. Animals of these three groups were treated daily at the doses of 250, 500 and 1000 mg/kg orally of POL-6 for 28 days. A G_5 group containing five male and five female rats was considered as a satellite group, POL-6 at the dose of 1000 mg/kg was given to the animals of the G_5 group for 28 days and then for next 14 days no treatment was given. The animals were sacrificed on day 43rd and aggravation, the persistence or the varying toxic effects were assessed. The G_1 group consisting of six male and six female rats was treated as a control group. Vehicle (1% sodium carboxymethyl cellulose) at

the dose of 1mL/ 100g body weight was given to the animals of the G_1 group for 28 days and on day 29, six animals (three males and three females) were sacrificed and other remaining six animals were considered as a control of the satellite group. The body weight change, water intake and feed consumption by the animals were recorded during the study period. Animals were observed twice a day for the sings of toxicity and morbidity/mortality [33].

Blood analysis

For hematological examinations, blood was collected at the end of the study from retro-orbital sinus of the animals in EDTA (ethylene–diamine–tetraacetate) coated vials and in 15 ml falcon tubes for serum separation. Hematological parameters like WBC (White blood cell count), MON (monocytes), NEU (Neutrophil), LYM (lymphocytes), EOS (eosinophil), RBC (Red blood cell count), BAS (Basophil), HGB (Hemoglobin), MCV (Mean corpuscular volume), ESR (Erythrocyte sedimentation rate), MCH (Mean corpuscular hemoglobin), platelet count and PCV (Packed cell volume) were determined as previously described. For separation of the serum, blood was centrifuged at 3000 RPM, 4°C temperature for 10 min and the obtained serum was used to assess the biochemical parameters like SGOT (serum glutamic–oxaloacetic transaminase), SGPT (serum glutamic–pyruvic transaminase), total bilirubin, blood urea, ALP (alkaline phosphatase), serum creatinine, direct bilirubin, total serum protein, globulin, albumin, A/G ratio (Albumin: globulin ratio) and total cholesterol using standard biochemical assay kit (Span Diagnostic Ltd., Gujarat, India) [34]

Organ weight and histopathology

Animals were sacrificed by cervical dislocation with anesthesia and detailed gross necroscopy was performed for both acute and sub-acute toxicity models. The weight of the organs like brain, heart, spleen, liver, thymus, kidneys, adrenals, testes/ovaries and, epididymis/uterus were documented and expressed in relative terms (g/100 g of total body weight). Heart, brain, liver, kidneys, lungs, pancreas, spleen, testes/ovaries were fixed in 10% NBF (Neutral buffered formalin). Testes were transferred to 10% NBF after fixing in modified Davidson's fluid for 48 h. The histological sections (5 μm) of the tissues were stained with H&E (Haematoxylin and eosin) method and examined for any change and cellular damage under the microscope [35].

Results and Discussion

Acute study

In acute safety evaluation, a single administration of POL-6 at a dose of 2000 mg/kg orally showed no behavioral signs of toxicity in rats when observed regularly for 30 min and periodically at 4 and 24hrs daily for 14 days. POL-6 administration did not show any signs of general activity (conscious state), muscle coordination, corneal reflexes, tremors, convulsions, straub, sedation, anesthesia, lacrimation, piloerection, ptosis, and salivation. POL-6 treated and parallel control group animals were similar in clinical manifestation**.** There were no changes in relative organ weights and water and food intake in rats. All animals survived during the 14 day study period and no treatment-related gross pathological changes were observed during the organ's necropsy examination on the 15^{th} day.

Sub-acute study

Effects on the general behavior and mortality

Oral administration of POL-6 at doses 250, 500 and 1000 mg/kg to the rats for 28 days did not produce any behavioral changes when compared to the control group. POL-6 administration in sub-acute safety assessment did not show any signs of general activity (conscious state), muscle coordination, corneal reflexes, tremors, convulsions, straub, sedation, anesthesia, lacrimation, piloerection, ptosis, and salivation. No mortality was observed during the study period.

Effects on the body weight, feed, and water intake

When compared to the control groups, POL-6 treated animals of both sexes did not show any significant changes in the feed consumption, body weights and water intake.

Haemato–biochemical parameters

Hematological examination showed that treatment with POL-6 did not produce any significant changes as white blood cell count, monocytes, lymphocytes, red blood cell count, neutrophil, basophil, eosinophil, hemoglobin count, mean corpuscular volume, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, erythrocyte rate, platelet count and packed cell volume, all parameters were in normal ranges as compared to the control group rats. Biochemical parameters like SGOT, ALP, SGPT, direct bilirubin, total bilirubin, blood urea,

total serum protein, serum creatinine, globulin, albumin, albumin: globulin ratio and total cholesterol were found to be normal when compared to the control group in both sexes.

Gross pathology

The relative (% body weight) organ weights of organs in POL-6 treated animals such as heart, brain, liver, spleen, thymus, kidneys, testes/ovaries, adrenals and epididymis /uterus did not show any significant difference when compared with those of the control group.

Histopathology

POL-6 treated animals did not show any gross pathological and microscopic variations when compared with the normal control. Brain showed normal glial cells in the histopathological examination (Fig. 9A) and heart showed normal myocardium morphology (Fig. 9A). No distinctive histological changes were seen in brain and heart at different doses of POL-6 treated rats. Normal hepatocytes with central and portal veins were observed during microscopic examination of the liver (Fig. 9B) and kidney showed normal nephrons without any interstitial bleeding, vacuolar degeneration of tubular cells, and glomerular congestion (Fig. 9B). No histological changes have been seen in liver and kidney of POL-6 treated rats as compared to the control group. Lungs of POL-6 treated rats appeared to be the same as that of the control with normal air passages as well as alveoli, without any interstitial thickening, disordered pulmonary alveoli structure, and basement membrane thickening and tearing (Fig. 9C). Histology of pancreas showed well–conserved pancreatic exocrine lobules containing packed acinar units, which are sharp–delimitated from islets of Langerhans (Fig. 9C). No characteristic histopathological changes were observed in lungs as well as in pancreas of POL-6 treated rats. Histology of spleen showed normal red pulp, white pulp distinction. Lymphoid follicles have normal mantle zones around them. There were no necrosis and hydropic degeneration observed in the splenetic sections (Fig. 9D) and sex organs of animals revealed focal spermatogenesis in male and normal ovarian follicles in female (Fig. 9D). No distinctive histopathological changes were seen in spleen and testis/ ovary of POL-6 treated rats.

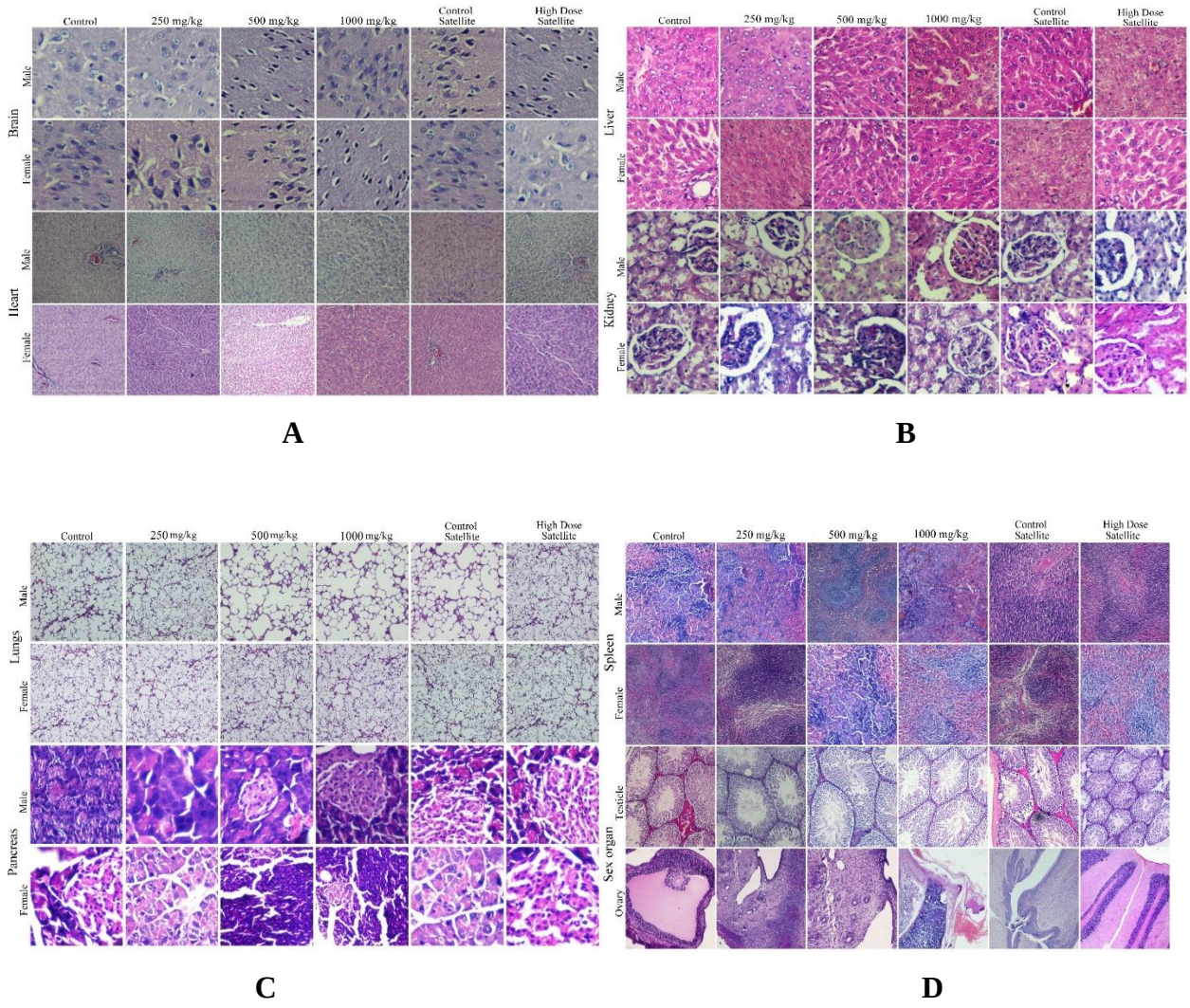


Fig. 9A Histology of brain (H&E x100) and heart (H&E x400) treated with POL-6 in the sub-acute toxicity study. **Fig. 9B**. Histology of liver and kidney treated with POL-6 in the sub-acute toxicity study (H&Ex100). **Fig 9C**. Histology of lungs and pancreas treated with POL-6 in the sub-acute toxicity study (H&E x100). **Fig.9D**. Histology of spleen and ovary/testis treated with POL-6 in the sub-acute toxicity study (H&E x100).

Objective 3: To screen the POL-6 in ethanol abstinence-induced behaviors like anxiety, depression and seizures in rats and to carryout comparative mRNA expression studies in control and ethanol abstinence animals for $GABA_A$ receptor and NMDA-glutamate receptor subunits.

Animals

Wistar rats (220-250g) were purchased from the National Institute of Pharmaceutical Education and Research, Mohali, Punjab, India and housed (two animals per cage) at Animal House, Jaypee University, Solan, H.P. They were acclimatized to laboratory conditions maintained at temperature 23±2ºC, controlled humidity conditions, and light and dark cycles (12:12 h). Animals were fed with food pellets diet (Aashirwad Industries, Chandigarh, India) and water *ad libitum.* Experimental procedures were carried out after the approval from the Institutional Animal Ethics Committee (3/GLG/2014/JUIT/IAEC) and as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) norms (1716/PO/a/13/CPCSEA).

Ethanol withdrawal study

Animals were divided randomly into 7 groups (n=6) and housed separately (Table 4.5). Group 1 received liquid diet; Group 2 was subjected to voluntarily ethanol intake for 15 days and received the vehicle 0.5% carboxymethyl cellulose (CMC) during ethanol withdrawal days (16^{th}, 17^{th} and 18^{th} day). Group 3, 4 and 5 were given alcohol treatment for 15 days and during ethanol withdrawal days received POL-6 (20, 50 and 100 mg/kg/, p.o.) once a day, respectively. Group 6 received alcohol treatment for 15 days and during ethanol withdrawal period diazepam (2mg/kg, p.o.) was administered once a day, Group 7 received alcohol treatment for 15 days and fluoxetine (5mg/kg, p.o.) was administered once a day during ethanol withdrawal period. Alcohol treatment was given to the animals as per described in the earlier studies. The alcohol fed animals were allowed to have free intake of 4.5%v/v ethanol on the 1^{st} day, 7.5%v/v ethanol on the 2^{nd} day and 9% v/v ethanol from third day to 15^{th} day with a 2 bottle choice paradigm (water vs ethanol). On 16^{th} day alcohol was withdrawn and liquid diet (alcohol free) was introduced to the alcohol fed animals while control group animals were continued on the same diet. As per earlier studies the peak level of withdrawal symptoms were observed on 3^{rd} day of withdrawal i.e. on 18^{th} day. Hence animals in the present study were subjected to the behavior parameters analysis on day 18^{th} only. One hour after the last dose of the drug treatment animals were individually tested for examining the anxiety on the elevated plus maze and light-dark test. Depression parameters were

studied by exposing the animals to the tail suspension test and forced swim test. Effect of alcohol withdrawal on locomotor activity was studied by exposing the animals to the actophotometer. To study the alcohol withdrawal seizures a sub convulsive dose of the Pentylenetetrazol (30mg/kg i.p.) was injected to the animals and seizure scoring was recorded. After behavior studies blood was withdrawn immediately through retro orbital of rats for hematological examination and serum was separated for examination of biochemical parameters. Body weight change and ethanol intake of the animals were recorded every day during the study and expressed as g/kg/day. After completion of the study animals were sacrificed by cervical dislocation and brain was isolated. The amygdala, hippocampus and prefrontal cortex were isolated from the rat brain for further analysis of variations in mRNA expression by RT-PCR (Fig. 10) [36-38].

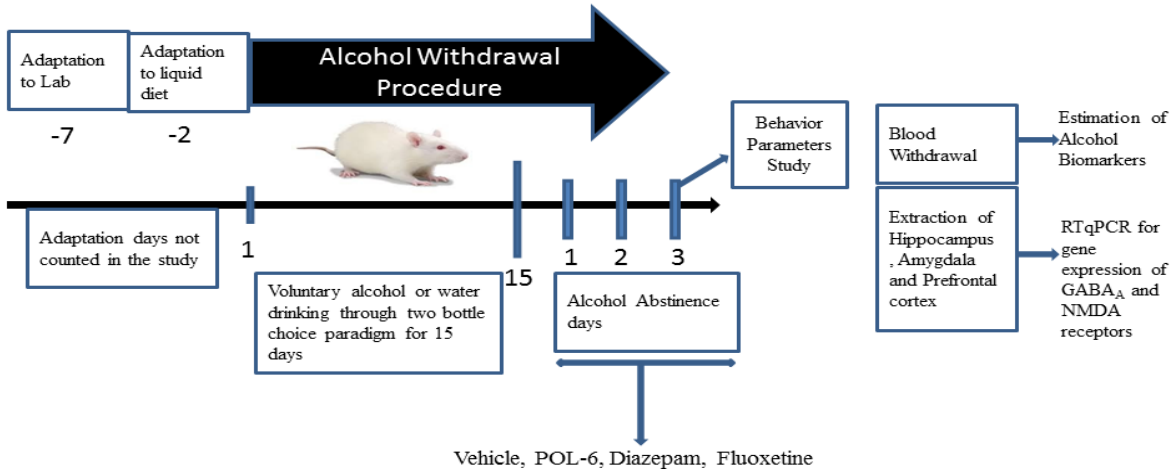


Fig 10: Experimental design for alcohol withdrawal study.

Blood alcohol concentration measurement

After consistent deliberate ethanol intake by the animals for 15 days blood was collected from the tail vein on day 15th and on 16th, 17th and 18th day (alcohol withdrawal period) in EDTA coated vials. Blood containing vials then centrifuged (1500×g) at 4 °C for 5-8 min. Plasma was stored at −20°C. BAC was determined by using bioassay systems' EnzyChrom ethanol assay kit.

Elevated plus maze model

The device consists of two open and two closed arms arranged opposite to one another and a central platform elevated at 50 cm. Animals were placed individually on the central platform

facing towards the open arm. The number of entries in the open arms, total time spent and the number of entries in the closed arms and total time spent were recorded by the observer for five minutes. The observations were carried out under red dim light [38].

Light and Dark Model

The model consists of two compartments one light and one dark in a rectangular box connected by an opening of 7.5 × 7.5 cm in between the wall. Animals were placed individually in the center of the light chamber facing towards the opening of the dark chamber. The number of entries among the light and dark chamber and the time spent in the light and dark chamber was recorded by the observer for five minutes [39].

Tail suspension test

In tail suspension test rats were suspended on the edge of a table 58 cm above the floor by the adhesive tape placed approximately 2-3 cm from the tip of the tail. The duration of immobility was recorded for a period of 5 min and observed to the extent of immobility versus active movement. The animal was considered to be immobile when it does not show any movement of the body and remain hanging passively [40].

Forced Swim Test

In forced swim test rats were individually forced to swim in a transparent vertical glass cylinder (45 cm high, 20 cm in diameter) filled with 27°C water to a depth of 20 cm. The duration of immobility of the animal (in sec) was measured for 6 min. 'Immobility' was defined as floating and treading water just enough to keep the nose above water. The water was changed for each animal [41].

Seizure scoring

Seizure scoring was also calculated after the administration of sub convulsive dose of Pentylenetetrazol (30mg/kg i.p.). Score 0: No response, Score 1: Ear and facial twitching, Score 2: Convulsive wave throughout the body, Score 3: Myoclonic jerks, Score 4: Clonic-tonic convulsions, turn over into side position, Score 5: Generalized clonic tonic seizures with loss of postural control, Score 6: Death [37].

Actophotometer

The locomotor behavior of the rats was accessed using actophotometer. In this animals were placed individually and the count was recorded when the animal cuts off light beam falling on the photocell is recorded digitally. The duration of the test was of 5 min. Counter was stopped after five min and the reading was noted. The rat was removed thereafter and the counter resets to zero. The same protocol was repeated for all rats

Biochemical analysis

After the behavior tests on day 18, animals were anesthetized with pentobarbital sodium (45 mg/kg) by intraperitoneal injection, and blood was collected from the retro-orbital sinus. Ethylene-diamine-tetra acetate coated vials were used to collect the blood sample for mean corpuscular volume (MCV) determination. Blood was also allowed to clot for 30 min at 25 °C and then centrifuged at 1500×g for 15 min at 4 °C to collect the serum samples for the biochemical estimation. ALT, AST, ALP, and GGT levels were assessed using commercially available biochemical assay kits [41].

Real-time quantitative polymerase chain reaction

On completion of the study protocol rats were sacrificed by cervical dislocation method. The prefrontal cortex, amygdala and hippocampus were extracted from the whole brain using an adult rat brain matrix (Kent scientific, USA) and placed in a sterile tube containing RNAlater solution (5 volumes). The tubes containing the brain tissues were further stored at -80^{o}C until further analysis. RT-PCR was carried out for studying mRNA expression of $GABA_A$ receptor subunits namely Gabra (1,2,3,4 and 5) and NMDA receptor subunits (Grin1, Grin2a, Grin2b). Trizol reagent was used for extracting total RNA from the brain tissues. RNA purity was evaluated on agarose gel (1.5%) in gel electrophoresis further quantification was done using a Nanodrop spectrophotometer (Thermo Scientific). Further 2 µg of total RNA was used for reverse transcription utilizing a verso RNA-to-cDNA synthesis kit (Thermo scientific). The RT-PCR analysis was performed using predesigned gene-specific primers for GABA and NMDA subunits using Primer Quest Tool (Table 8). Bio-Rad CFX96™ RT-PCR detection system was used for RT-PCR amplification using SYBR green dye. Total reaction mixer volume was 12.5 µl comprising of 2.5 pM of each primer and cDNA template (1 µl). Relative expression levels of the target genes were estimated using housekeeping gene rat Beta-actin as an endogenous

control. The thermal cycle profile for 40 cycles to amplify cDNA was as: 95 °C for 3 min; 95 °C for 15 s, 50 °C – 57 °C for 60 s and 72 °C for 2 min. The gene expression of the targeted genes was calculated by using double delta threshold cycle (ΔΔCT) method.

Table 8: Genes and primer sequences

S. N.	Gene	Primer sequence
1.	Gabra1	FP: GCCCTCCCAAGATGAACTTA RP: AGTTACACGCTCTCCCAAGC
2.	Gabra2	FP: ACCTTCTTTCACAACGGGAA RP: GGAAAGTCCTCCAAGTGCAT
3.	Gabra3	FP: CAAGCATGCCCCAGATATTC RP: CAGTCACTGCATCTCCAAGC
4.	Gabra4	FP: CCGTATCCTGGACAGTTTGC RP: ACATCAGAAACGGGTCCAAA
5.	Gabra5	FP: TGAGACCAATGACAACATCA RP: TAGATGTCTGTTCGCACCTG
6.	Grin1	FP: TTCAAGAGGGTGCTGATGTC RP: CCATTGTAGATGCCCACTTG
7.	Grin2a	FP: GTGATCGTGCTGAACAAGGA RP: CTGAGGTGGTTGTCATCTGG
8.	Grin2b	FP: CCGAAGCTGGTGATAATCCT RP: TCAGATGGTCATCCTCTTGC

Results and Discussion

Ethanol consumption and body weight changes of the animals

Ethanol fed group animals were given free access of ethanol 4.5% v/v on the 1st day, 7.5% on the 2nd day and then maintained on 9% v/v ethanol from the 3rd day to 15th day. Ethanol intake by individual animal in ethanol fed groups was recorded daily for 15 days and calculated as g/kg/day. Daily ethanol consumption in ethanol fed groups varies between 13.57±1.85 to 17.12±1.34 g/kg during the exposure to 9% ethanol. No significant difference in ethanol intake

was noticed among the ethanol fed groups. An increase in the body weight of approximately 11.2 % in control group animals and 6.4% in ethanol fed animals was observed over the initial body weight at the end of the study. Changes in animal's body weight observed during the study are depicted in Table 9.

Table 9: Body weight changes of the animals during acute toxicity study. Values are means ± SD; g = gram

Body Weight (g)			
Groups	**Beginning of the study**	**End of the study**	**Body weight change (%)**
Control	215.71 ± 4.56	239.86 ± 5.06	11.2%
Alcohol fed	213.30 ± 5.49	226.95 ± 6.01	6.4%

Blood alcohol concentration measurement

Blood alcohol concentration (BAC) was measured on day 15^{th} and on alcohol withdrawal days 16^{th}, 17^{th} and 18^{th} and was expressed as mean ± SEM. On day 15^{th} of the study, BAC was found to be 111.6 ± 8.151 mg %. Very low concentration of alcohol was observed in the animals after 24 hrs of alcohol withdrawal (8.012 ± 1.325 mg %), 2.017 ±0.285 mg % after 48 hrs of alcohol withdrawal, 0.00 ± 0.00 mg % after 72 hrs of alcohol withdrawal.

Anxiety tests

Elevated plus maze

When tested on the EPM ethanol fed animals revealed a significant decrease ($p<0.001$) in the time spend and in number of entries into the open arms when compared to the normal control group. A significant increase ($p<0.001$) in the time spent and in number of entries into the closed arms was also observed in the ethanol fed animals when compared to the normal group animals (Fig. 11 A, B, C and D). These findings revealed the development of anxiety in the ethanol withdrawal animals. Treatment with POL-6 (20, 50 and 100mg/kg, oral) and diazepam (2mg/kg) for three consecutive days produced a significant ($p<0.001$) increase in the time spend and in

number of entries into the open arms and significant decrease ($p<0.001$) in the time spend and in number of entries into the closed arms when compared to the disease control rats (Fig. 11 A, B, C and D).

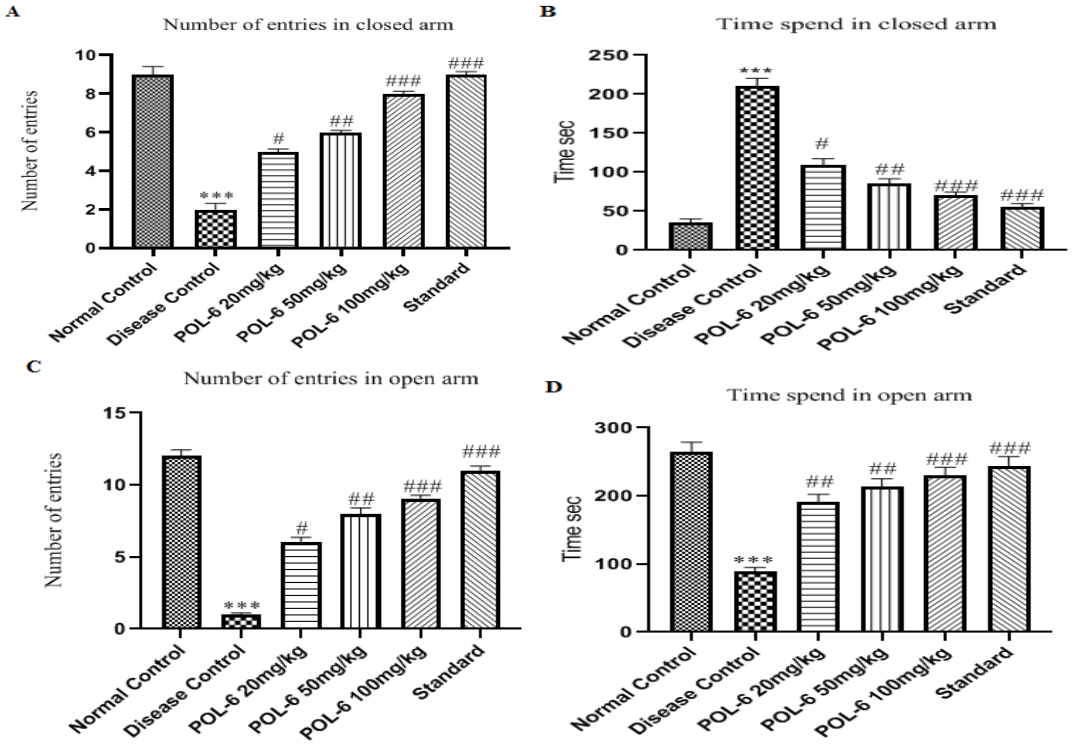


Fig 11: Effect of drug treatment on ethanol withdrawal anxiety when tested on elevated plus maze in rats. (Fig. A) Number of entries in the closed arm. (Fig. B) Time spent in the closed arm. (Fig. C) Number of entries in the open arm. (Fig. D) Time spent in the open arm. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Compared to normal control); #$p<0.05$, ##$p<0.01$, ###$p<0.001$ (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

Light and dark model

When tested on the light and dark model ethanol fed animals revealed a significant decrease ($p<0.001$) in the time spent and in number of entries into the light chamber of the light and dark model when compared to the normal control animals. A significant increase ($p<0.001$) in the time spend and in number of entries into the dark chamber of light and dark model was also noticed in the ethanol fed animals when compared to the normal control animals (Fig. 12 A, B, C

D). Similar to the EPM, the results from the light and dark model revealed the development of anxiety in the ethanol withdrawal animals. Treatment with POL-6 (20, 50 and 100mg/kg, oral) and diazepam (2mg/kg) for three consecutive days produced a significant ($p<0.001$) increase in the time spend and in number of entries into the light chamber and significant decrease ($p<0.001$) in the time spend and in number of entries into the dark chamber when compared to the disease control rats (Fig. 12 A, B, C D).

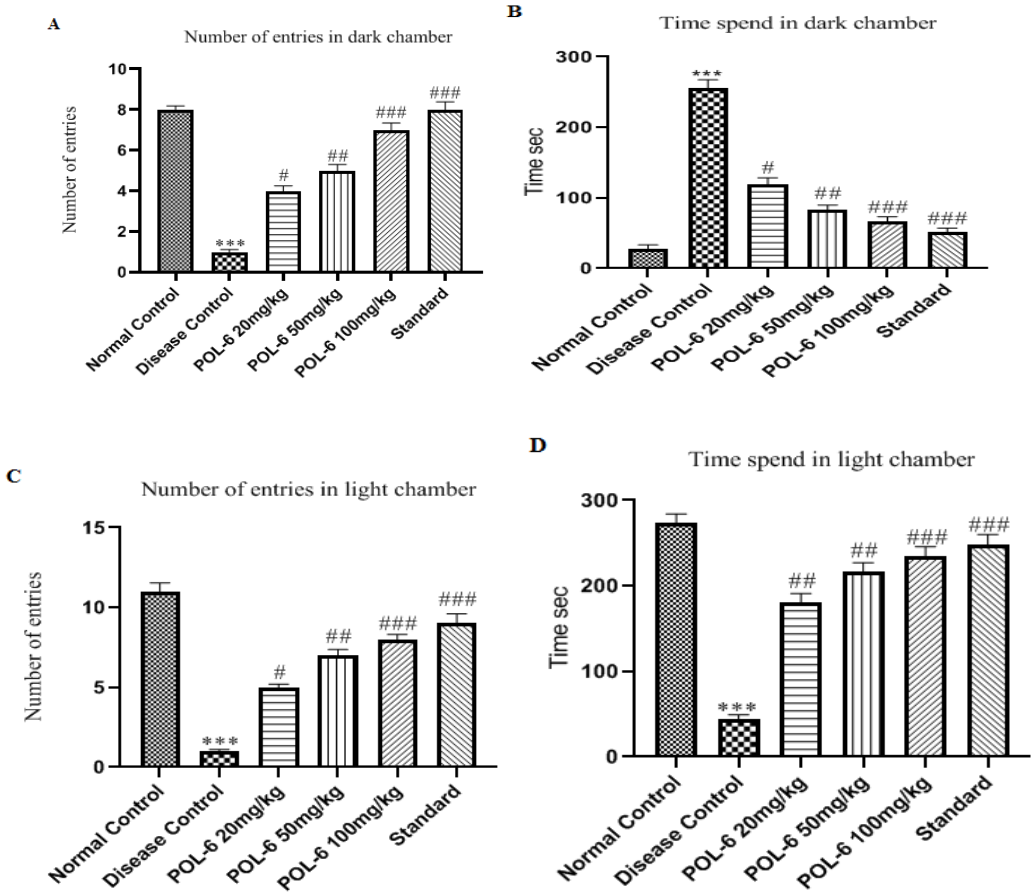


Fig 12: Effect of drug treatment on ethanol withdrawal anxiety when tested on light and dark model in rats. (Fig. A) Number of entries in the dark chamber (Fig. B) Time spend in the dark chamber.(Fig. C) Number of entries in the light chamber (Fig. D) Time spend in the light chamber. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Compared to normal control); #$p<0.05$, ##$p<0.01$, ###$p<0.001$ (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

Depression tests

Rats were subjected to the depression paradigm on the 3rd day of alcohol withdrawal (18th day of the study). Fluoxetine (5 mg/kg i.p) was used as a standard drug during depression paradigm study.

Forced swim test

Ethanol-fed rats revealed a significant increase ($p<0.001$) in the immobility time when compared to the immobility time of the normal group rats in the FST (Fig 13). The results from the FST indicate the development of depression in the ethanol withdrawal animals. Treatment with POL-6 (20, 50 and 100mg/kg, oral) and fluoxetine (5 mg/kg i.p) for three consecutive days produced the significant ($p<0.001$) decrease in the immobility time when compared to the immobility time of the disease control animals in the FST (Fig 13).

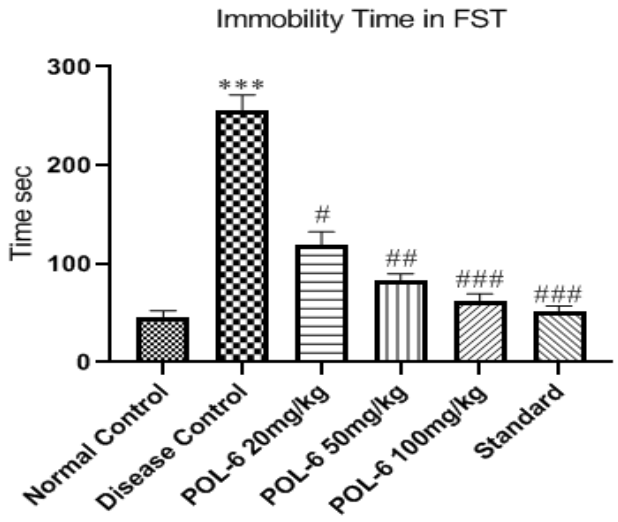


Fig 13: Effect of drug treatment on ethanol withdrawal depressive like behavior when tested in forced swim test in rats. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Compared to normal control); #$p<0.05$, ##$p<0.01$, ###$p<0.001$ (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

Tail suspension test

In TST, ethanol-fed rats revealed a significant increase ($p<0.001$) in the immobility time when compared to the immobility time of the normal group animals (Fig 14). Similar to the FST, the results from the TST indicate the development of depression in the ethanol withdrawal animals.

Treatment with POL-6 (20, 50 and 100mg/kg, oral) and fluoxetine (5 mg/kg i.p) for three consecutive days produced the significant ($p<0.001$) decrease in the immobility time when compared to the immobility time of the disease control animals in the TST (Fig 14).

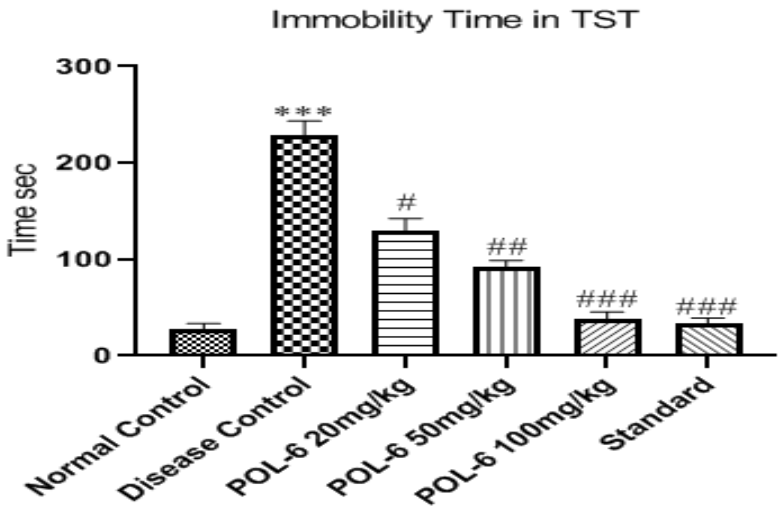


Fig 14: Effect of drug treatment on ethanol withdrawal depressive like behavior when tested in forced swim test in rats. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Compared to normal control); #$p<0.05$, ##$p<0.01$, ###$p<0.001$ (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

Seizures test

Effect of POL-6 was tested on the alcohol withdrawal seizures in the animals on the 3rd day of alcohol withdrawal (18th day of the study). For studying alcohol withdrawal seizures a sub convulsive dose of the Pentylenetetrazol (30mg/kg i.p.) was administered to the alcohol fed animals and seizure scoring was recorded thereafter.

Seizure scoring

Pentylenetetrazol administration at sub convulsive dose (30mg/kg i.p.) causes an increase in the seizure severity and frequency in the ethanol withdrawal rats. No seizures were observed in the normal control animals after Pentylenetetrazol administration at sub convulsive dose (30mg/kg i.p.). Treatment with POL-6 (20, 50, 100 mg/kg, oral) and Diazepam (2 mg/kg) showed decreased seizure severity and frequency in the alcohol fed animals. The seizures were recorded

as; Score 0: No reaction, Score 1: Ear and facial jerking Score 2: convulsive wave all through the body, Score 3: Myoclonic jerks, Score 4: Clonic tonic spasms, transform over into side position, Score 5: Generalized clonic tonic seizures with loss of postural control, Score 6: Death. The results of the seizure scoring are showed in the Table 10.

Table 10: Seizure severity in the animals.

GROUP 1	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	6	0	0	0	0	0	0
GROUP 2	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	0	6	6	6	6	6	3
GROUP 3	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	0	3	6	3	0	0	0
GROUP 4	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	0	5	6	3	0	0	0
GROUP 5	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	0	6	6	2	0	0	0
GROUP 6	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	0	4	6	2	0	0	0

Actophotometer

When animals were tested on the actophotometer during the alcohol withdrawal period the ethanol-fed rats showed a significant ($p<0.001$) locomotor hyperactivity when compared with the normal group animals (Fig. 5.21). The results from the actophotometer indicate the development of locomotor hyperactivity in the ethanol withdrawal animals. Treatment with POL-6 (20, 50 and 100mg/kg, oral) and diazepam (2mg/kg) for three consecutive days significantly ($p<0.001$) reduced the ethanol withdrawal locomotor hyperactivity when compared to the disease control animals.

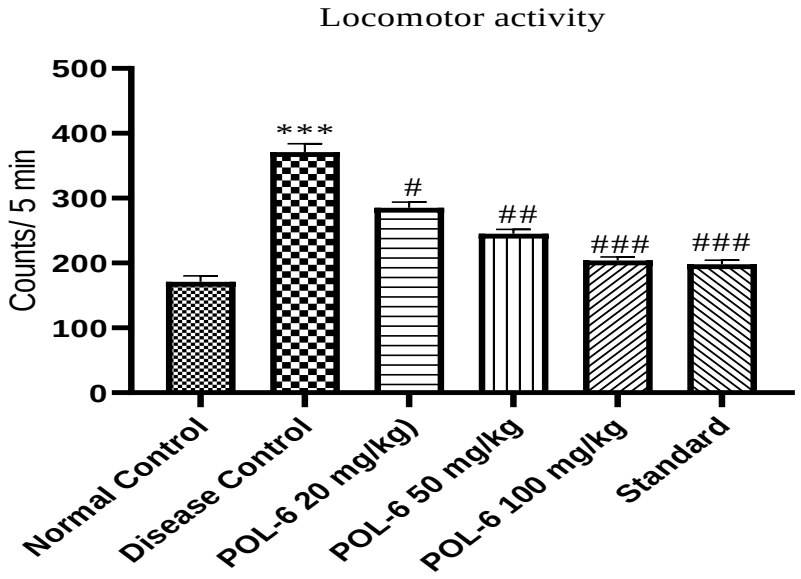


Fig 15: Effect of drug treatment on alcohol withdrawal locomotor hyperactivity when tested on Actophotometer. *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

Biochemical parameters evaluation

Liver function tests

The effect of POL-6 was explored on the traditional alcohol liver markers like GGT, SGOT and SGPT. Alcohol administration in alcohol fed animals for following 15 days significantly (p<0.001) elevated the levels of SGOT, SGPT and GGT when compared to the normal control animals. POL-6 (20, 50, 100 mg/kg, oral) and diazepam (2mg/kg) treatment for following 3 days significantly (p<0.001) reversed the elevated levels of GGT, SGOT and SGPT when compared to the disease control animals. However alcohol, POL-6 and diazepam treatment did not bring about any significant change in the total bilirubin, albumin and globulin levels when compared to the normal group animals. The results of alcohol liver markers are shown in the Table 11.

Table 11: Effect of drug treatment on liver markers. [a]p<0.001 (Compared to normal control); [b]p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

Parameters	Normal Control	Disease Control	POL-6 (20 mg/kg)	POL-6 (50 mg/kg)	POL-6 (100 mg/kg)	Diazepam (2 mg/kg)
Total Bilirubin (mg/dl) (0.1-0.55)	0.50±0.02	0.51±0.04	0.49±0.10	0.47±0.08	0.42±0.19	0.51±0.06
SGOT(µ/l) (20-70)	54.02±10.66	109±11.71[a]	83.5±9.42	73.1±16.14[b]	57±13.48[b]	53±8.80[b]
SGPT (µ/l) (10-30)	26.33±7.44	60.5±9.83[a]	48.2±6.91[b]	40.8±7.92[b]	33.23±10.4[b]	34.34±7.2[b]
Albumin gm(%) (2.8-4.8)	4.5±0.14	4.2±0.23	4.2±1.42	4.3±1.01	4.4±0.18	4.5±0.46
Globulin gm(%) (1.8-3.2)	3.1±0.04	3.2±0.18	3.1±0.41	3.0±1.63	3.1±1.68	3.2±0.41
GGT(iu/l) (10-70)	18.25±1.12	78.9±1.8[a]	67.10±1.79	51.7±1.43[b]	29.4±0.79[b]	26.8±0.42[b]

Kidney function tests

Alcohol treatment for 15 days and POL-6 (20, 50, 100mg/kg, oral) and diazepam (2mg/kg) treatment in alcohol fed rats for following 3 days did not produce any significant change in the kidney function tests like blood urea, serum creatinine and serum uric acid when compared to the normal group animals. The results of the kidney function tests are depicted in the Table 12.

Table 12: Effect of drug treatment on Kidney function. $^{\#}p<0.001$ (Compared to normal control); *****p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

Groups	Blood urea (mg/dl)(10-32)	Serum creatinine (mg/dl)(0.2-0.8)	Serum uric acid (mg/dl)(3-6)
Normal Control	28.08±2.55	0.80± 0.13	3.66±0.19
Disease Control	27.98±3.81	0.81±0.03	3.86±0.36
POL-6 (20 mg/kg)	29.48± 2.51	0.82±0.07	4.12±0.27
POL-6 (50 mg/kg)	26.03± 2.81	0.80±0.04	3.91±0.31
POL-6 (100 mg/kg)	29.13± 1.47	0.80±0.02	3.08±0.44
Diazepam (2mg/kg)	27.00± 2.20	0.81± 0.06	3.23±0.54

RT-PCR analysis

Effect of drug treatment on mRNA expression in $GABA_A$ receptor and NMDA-glutamate receptor subunits in Pre-frontal Cortex, Amygdala and Hippocampus was analyzed by RT-PCR.

Prefrontal cortex

$GABA_A$ subunits

Fig. 16 A, B, C, D and E demonstrates the influences of POL-6 on the altered expression of $GABA_A$ receptor subunits in the rats' prefrontal cortex. Two-way ANOVA represented the influences of POL-6 on the relative expression of Gabra (1-5) in the prefrontal cortex. Dunnett's multiple comparison test revealed that the relative gene expression of Gabra1, Gabra2, Gabra3, Gabra4 and Gabra 5 were significantly ($p<0.001$) down regulated in the disease control rats when compared with the normal control rats. Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly ($p<0.001$) normalized the down regulated genes Gabra1, Gabra 2, Gabra 3 and Gabra 5 when compared to the disease control rats. No significant changes were detected in the expression of Gabra 4 on the treatment with POL-6 (20, 50 and 100 mg/kg, oral).

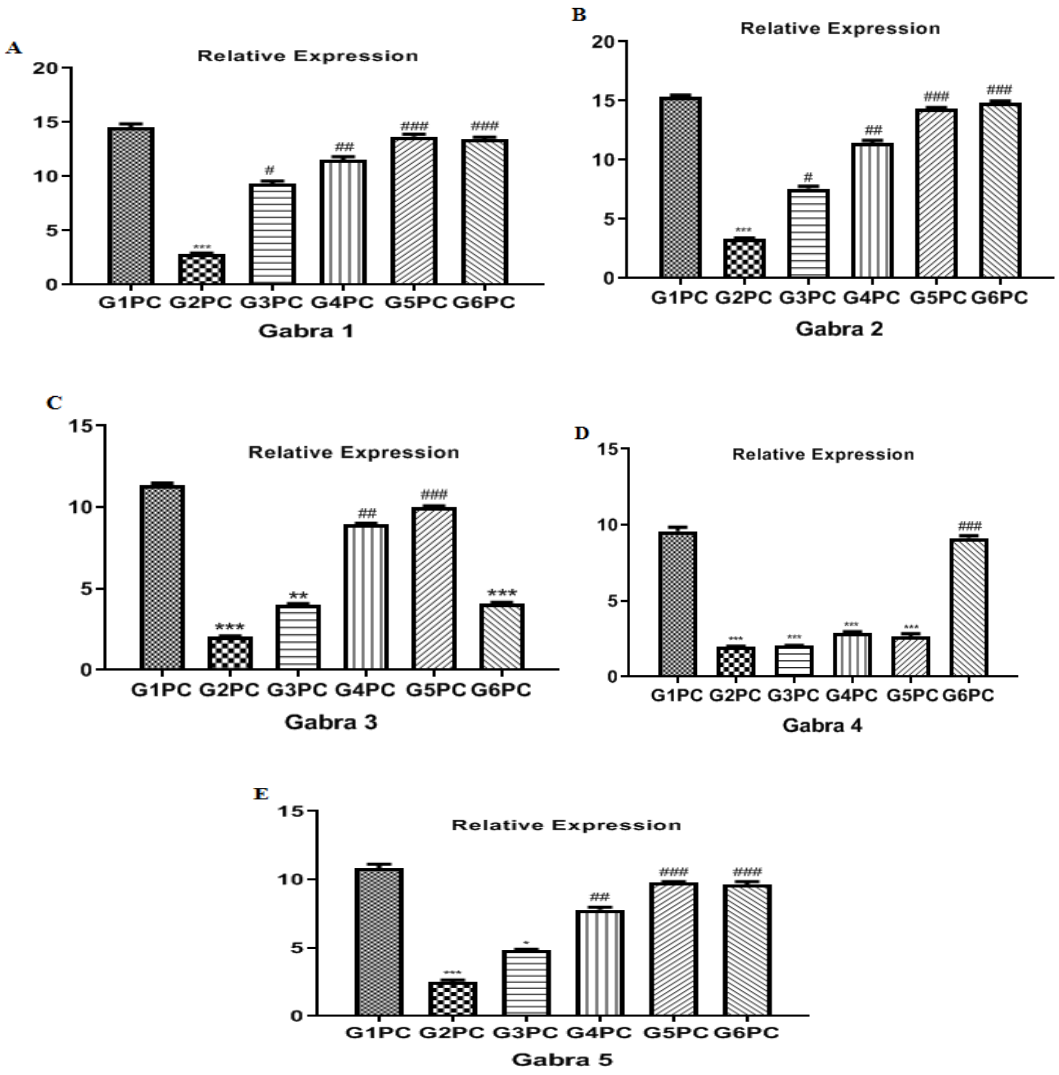


Fig 16: Effect of drug treatment on the relative mRNA expression of $GABA_A$ subunits in prefrontal cortex (PC) of the rats. (Fig. A) Gabra 1 (Fig. B) Gabra 2 (Fig. C) Gabra 3 (Fig. D) Gabra 4 (Fig. E) Gabra 5**.** *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

Diazepam (2mg/kg) treatment significantly (p<0.001) normalized the down regulated genes Gabra1, Gabra 2, Gabra4 and Gabra 5 as compared to the disease control rats however; no significant changes were detected in the expression of Gabra 3 on the treatment with Diazepam (2mg/kg).

NMDA subunits

Fig. 17 A, B and C demonstrates the influences of POL-6 on the altered expression of NMDA subunits in the rats' prefrontal cortex. Two-way ANOVA represented the influences of POL-6 on the relative expression of Grin1, Grin2a and Grin2b in the prefrontal cortex. Dunnett's multiple comparison test revealed that the relative expression of Grin1, Grin2a and Grin2b were significantly ($p<0.001$) up regulated in the disease control rats when compared to the normal control rats. Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly ($p<0.001$) normalized the up regulated genes Grin1 and Grin2a when compared to the disease control rats.

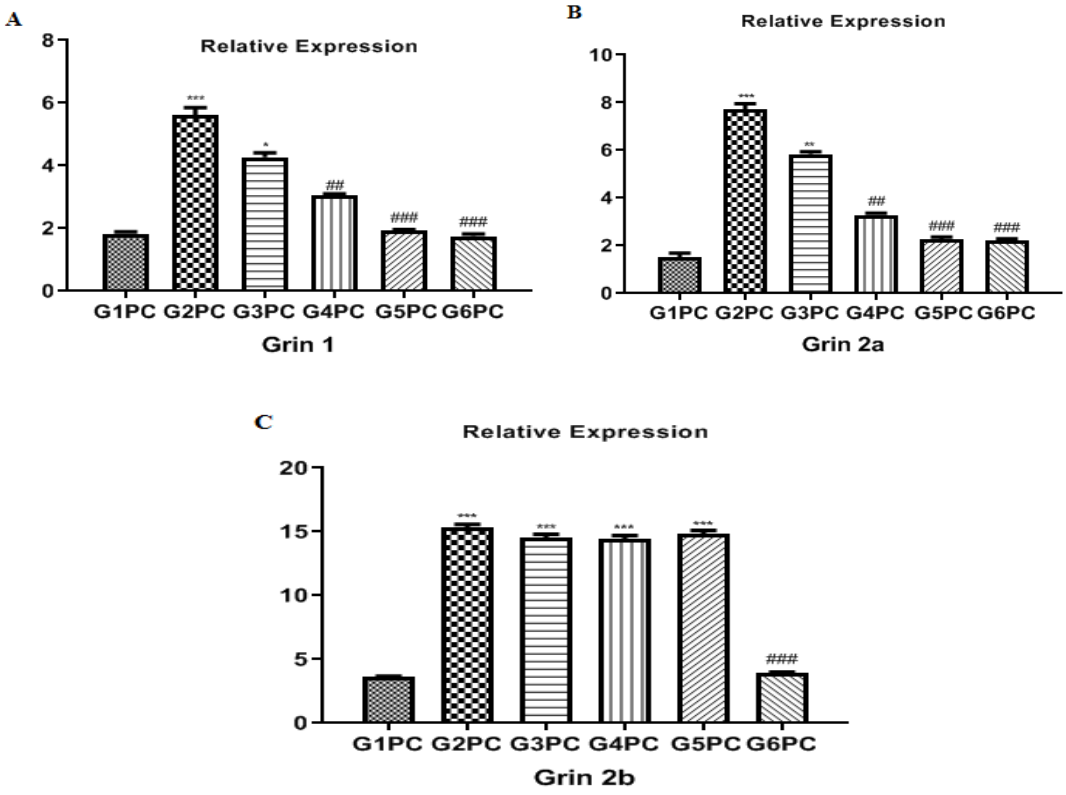


Fig 17: Effect of drug treatment on the relative mRNA expression of NMDA subunits in prefrontal cortex (PC) of the rats. (Fig. A) Grin 1 (Fig. B) Grin 2a (Fig. C) Grin 2b *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Compared to normal control); #$p<0.05$, ##$p<0.01$, ###$p<0.001$ (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

No significant changes were detected in the gene expression of Grin2b on the treatment with POL-6 (20, 50 and 100 mg/kg, oral). Diazepam (2mg/kg) treatment significantly ($p<0.001$) reversed the down regulated genes Grin1, Grin2a and Grin2b in comparison to the disease control rats.

Amygdala

$GABA_A$ subunits

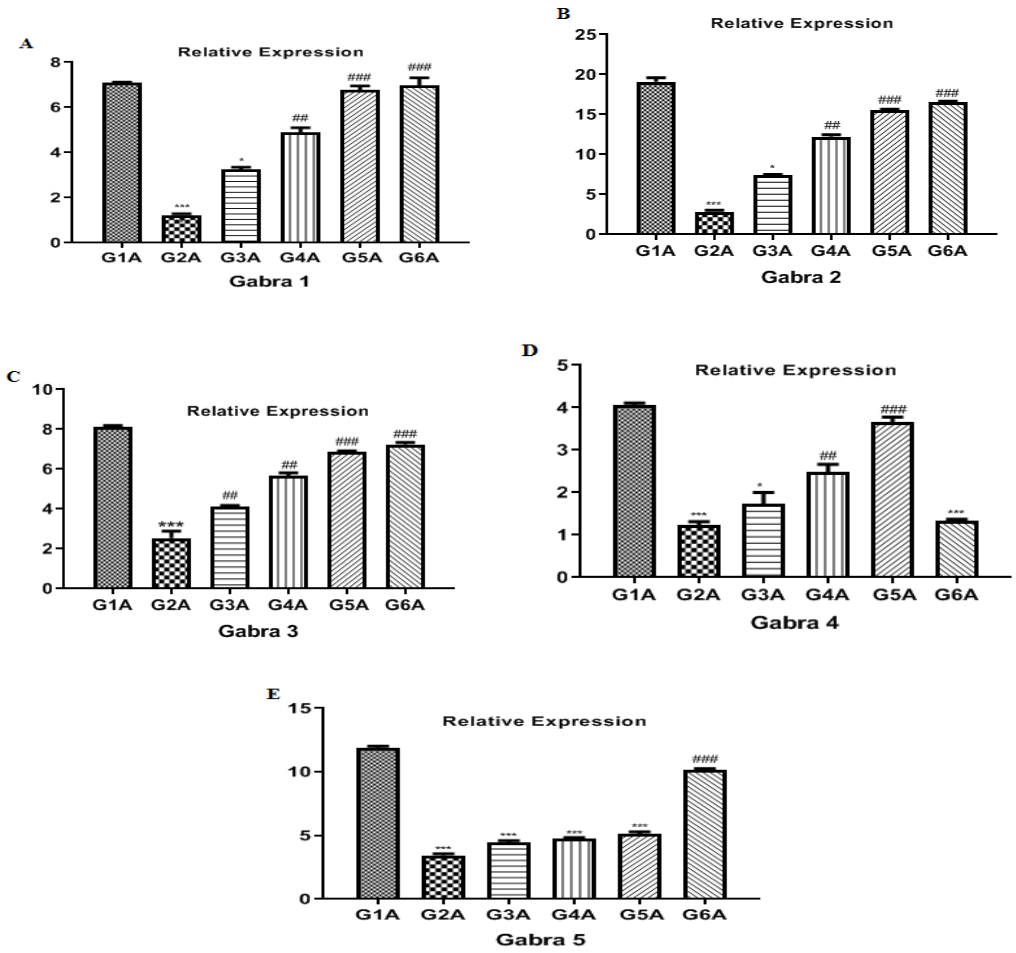


Fig 18: Effect of drug treatment on the relative mRNA expression of $GABA_A$ subunits in amygdala (A) of the rats. (Fig. A) Gabra 1 (Fig. B) Gabra 2 (Fig. C) Gabra 3 (Fig. D) Gabra 4 (Fig. E) Gabra 5**.** *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Compared to normal control); #$p<0.05$, ##$p<0.01$, ###$p<0.001$ (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

Fig. 18 A, B, C, D, and E demonstrates the influences of POL-6 on the altered expression of $GABA_A$ receptor subunits in rats' amygdala. Two-way ANOVA represented the influences of POL-6 on the relative expression of Gabra1, Gabra2, Gabra3, Gabra4 and Gabra 5 in the amygdala. Dunnett's multiple comparison tests showed that the relative expression of Gabra1, Gabra2, Gabra3, Gabra4 and Gabra 5 were significantly ($p<0.001$) down regulated in the disease control rats when compared to the normal control rats. Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly ($p<0.001$) normalized the down regulated genes Gabra1, Gabra 2, Gabra 3 and Gabra 4 in comparison to the disease control rats. No significant changes were detected in the gene expression of Gabra 5 on the treatment with POL-6 (20, 50 and 100 mg/kg, oral). Diazepam (2mg/kg) treatment significantly ($p<0.001$) normalized the down regulated genes Gabra1, Gabra 2, Gabra3 and Gabra 5 in comparison to the disease control rats however; no significant changes were detected in the gene expression of Gabra 4 on the treatment with Diazepam (2mg/kg).

NMDA subunits

Fig. 19 A, B and C demonstrates the influences of POL-6 on the altered expression of NMDA subunits in rats' amygdala. Two-way ANOVA represented the influences of POL-6 on the relative expression of Grin1, Grin2a and Grin2b in the amygdala.

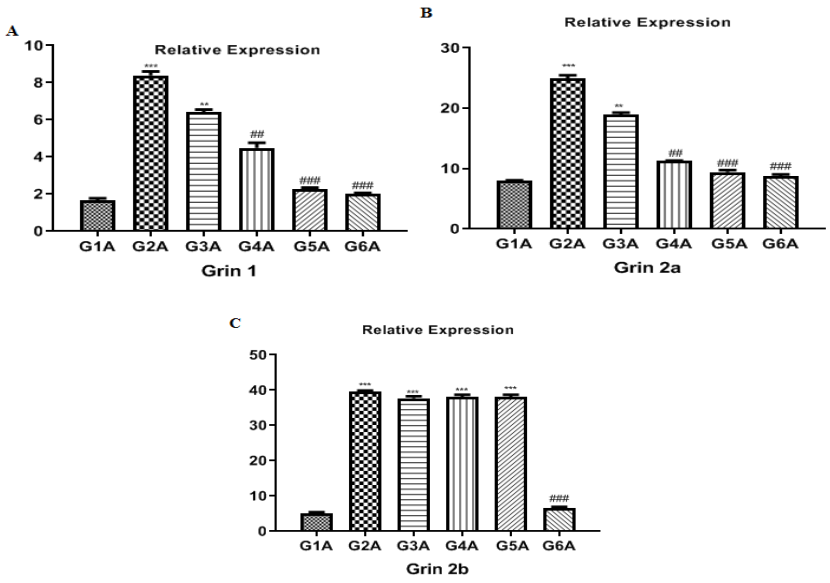


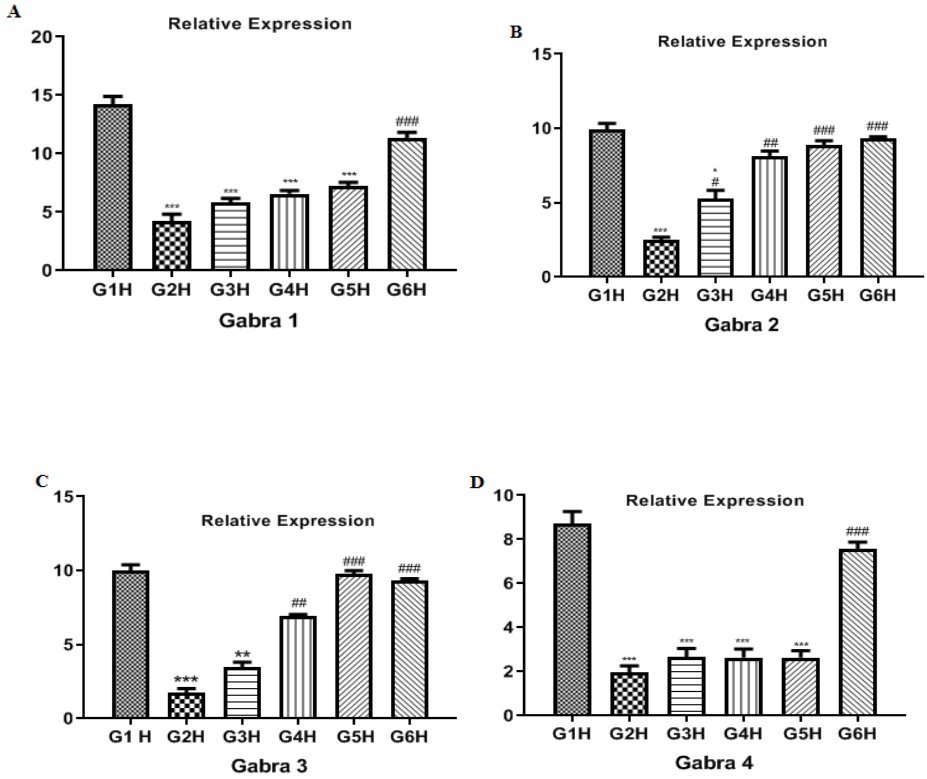
Fig 19: Effect of drug treatment on the relative mRNA expression of NMDA subunits in amygdala (A) of the rats. (Fig. A) Grin 1 (Fig. B) Grin 2a (Fig. C) Grin 2b *$p<0.05$, **$p<0.01$,

***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

Dunnett's multiple comparison tests showed that the relative expression of Grin1, Grin2a and Grin2b were significantly (p<0.001) up regulated in the disease control rats when compared to the normal control rats. Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly (p<0.001) normalized the up regulated genes Grin1 and Grin2a in comparison to the disease control rats. No significant changes were detected in the gene expression of Grin2b on the treatment with POL-6 (20, 50 and 100 mg/kg, oral). However, Diazepam (2mg/kg) treatment significantly (p<0.001) reversed the down regulated genes Grin1, Grin2a and Grin2b in comparison to the disease control rats.

Hippocampus

$GABA_A$ subunits



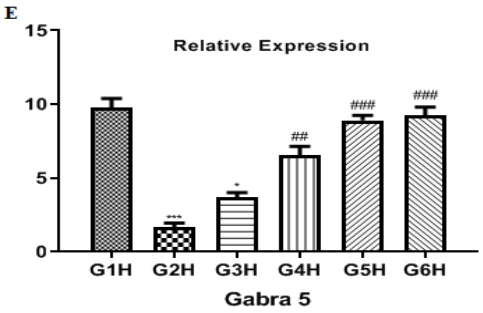


Fig 20: Effect of drug treatment on the relative mRNA expression of $GABA_A$ subunits in hippocampus (H) of the rats. (Fig. A) Gabra 1 (Fig. B) Gabra 2 (Fig. C) Gabra 3 (Fig. D) Gabra 4 (Fig. E) Gabra 5**.** *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

Fig. 20 A, B, C, D and E demonstrates the influences of POL-6 on the altered expression of $GABA_A$ receptor subunits in the rats' hippocampus. Two-way ANOVA represented the influences of POL-6 on the relative expression of Gabra1, Gabra2, Gabra3, Gabra4 and Gabra 5 in the hippocampus. Dunnett's multiple comparison tests showed that the relative expression of Gabra1, Gabra2, Gabra3, Gabra4 and Gabra 5 were significantly ($p<0.001$) down regulated in the disease control rats when compared to the normal control rats. Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly ($p<0.001$) normalized the down regulated genes Gabra 2, Gabra 3 and Gabra 5 in comparison to the disease control rats. No significant changes were detected in the expression of Gabra 1 and Gabra 4 on the treatment with POL-6 (20, 50 and 100 mg/kg, oral). Diazepam (2mg/kg) treatment significantly ($p<0.001$) normalized the down regulated genes Gabra1, Gabra 2, Gabra3, Gabra4 and Gabra 5 in comparison to the disease control rats.

NMDA subunits

Fig. 21 A, B and C demonstrates the influences of POL-6 on the altered expression of NMDA subunits in the rat's hippocampus. Two-way ANOVA represented the influences of POL-6 on the relative expression of Grin1, Grin2a and Grin2b in the hippocampus. Dunnett's multiple comparison tests showed that the relative expression of Grin1, Grin2a and Grin2b were

significantly ($p<0.001$) up regulated in the disease control rats when compared to the normal control rats. Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly ($p<0.001$) normalized the up regulated gene Grin1 in comparison to the disease control rats. No significant changes were detected in the expression of Grin 2a and Grin2b on the treatment with POL-6 (20, 50 and 100 mg/kg, oral). However, Diazepam (2mg/kg) treatment significantly ($p<0.001$) reversed the down regulated genes Grin1, Grin2a and Grin2b in comparison to the disease control rats.

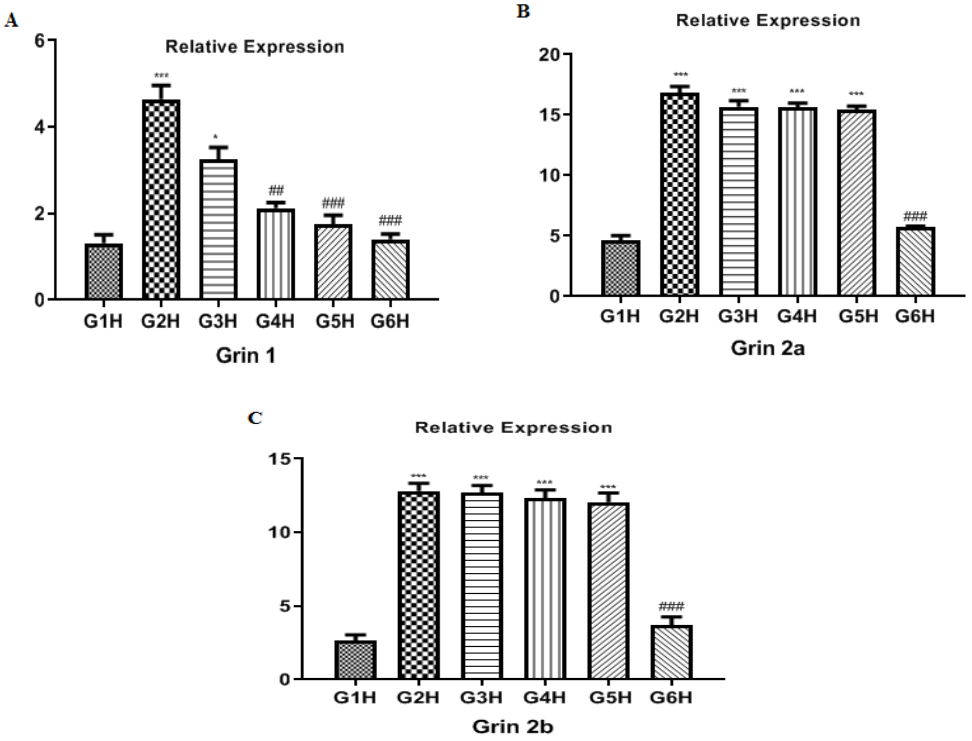


Fig 21: Effect of drug treatment on the relative mRNA expression of NMDA subunits in hippocampus (H) of the rats. (Fig. A) Grin 1 (Fig. B) Grin 2a (Fig. C) Grin 2b *$p<0.05$, **$p< 0.01$, ***$p<0.001$ (Compared to normal control); #$p<0.05$, ##$p<0.01$, ###$p<0.001$ (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

Conclusion

The current study elucidates the protective effect of Polyherbal preparation (POL-6) against the ethanol withdrawal syndrome. POL-6 was found to be safe in rats when administered for 28 days in sub-acute toxicity studies. Acute and sub-acute toxicity studies of POL-6 shows that the LD_{50} of POL-6 is more than 1000mg/kg. POL-6 showed significant protective effect in alcohol withdrawal anxiety, depression, seizures and locomotor hyperactivity when tested through animal behavioral models. Gene expression studies on the isolated brain tissues shows that POL-6 normalizes the GABAergic and NMDA transmission in the amygdala, pre-frontal cortex and hippocampus of the rats and inhibits the ethanol withdrawal behaviors. Therefore we concluded that POL-6 may have therapeutic potential for treating ethanol-type dependence as it suppresses ethanol withdrawal signs and symptoms.

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Publications

1. **L. Sharma**, A. Sharma, G.L. Gupta. "Standardization of a polyherbal preparation (POL–6) for treatment of oxidative, inflammatory and immune disorders" *International Journal of Pharmacy and Pharmaceutical Sciences*. vol. 8(4), pp. 129-134, 2016.
2. **L. Sharma**, A. Sharma, G.L. Gupta, G.S. Bisht. Acute and Sub-acute. "Oral Toxicity Assessment of a Standardized Polyherbal Preparation POL-6 in Rats", *The Natural Products Journal*. vol. 9(3), pp.207-216, 2019.
3. **L. Sharma**, A. Sharma, G.L. Gupta, G.S. Bisht. "Protective effect of Ocimum sanctum Linn. leaf extract on ethanol withdrawal syndrome in Wistar rats", *Asian Pacific Journal of Tropical Medice*, vol. 11(8), pp. 423-428, 2018.
4. **L. Sharma**, A. Sharma, G.L. Gupta, G.S. Bisht. "Pharmacological Evaluation *of Bacopa monnieri* Extract against Depressive like Behavior by Ethanol Withdrawal in Rats." *Pharmacognosy Journal.* vol. 10(6), pp. s73-78, 2018.
5. **L. Sharma**, A. Sharma, G.L. Gupta, G.S. Bisht. "A standardized polyherbal preparation POL-6 normalizes GABAergic transmission in rats's amygdla and prevents ethanol withdrawal anxiety like behavior" (Due for submission).

Attended Conferences

1. **L.Sharma**, G.L.Gupta, G.S. Bisht "*Centella asiatica* extract normalizes GABAnergic transmission in the rats amygdala and attenuates ethanol withdrawal anxiety like behavior associated with ethanol abstinence" 18th World Congress of Basic and Clinical Pharmacology (WCP2018) organized by IUBHAR and Japanese Pharmacological Society, held at **Kyoto International Conference Centre, Kyoto, Japan**, July 1-6th 2018.
2. **L.Sharma**, G.L.Gupta, G.S. Bisht "Authorship: Professional, ethical and operational issues" 6th World conference on research integrity, organized by The University of Hong Kong and WCRI, held at **The University of Hong Kong, Hong Kong**, June 2-5th 2019.
3. **L. Sharma**, A. Sharma, G.L. Gupta "Effect of Polyherbal extract on the alcohol deprivation effects following long term voluntary alcohol consumption in rats." IPSCON-2015 organized by Saurashtra university and Indian Pharmacological Society held at **Saurashtra university, Rajkot, Gujarat, India, December** 18-20th 2015.

4. **L.Sharma**, A.Sharma, G.L. Gupta "Protectve effect of *Hypericum perforatum* in ethanol withdrawal syndrome followed by long term ethanol consumption in rats. IPSCON-2014 oraganised by Indian Pharmacological society, Gauhati medical college and NIPER held at **Guwahati medical college, Guwahati, Assam, India, December** 28-30th 2014.
5. **L. Sharma**, A. Sharma, G.L. Gupta "*Oscimum sanctum* Linn. extract normalizes GABAnergic transmission in the rat amygdala and inhibits the ethanol withdrawal induced anxiety like behaviour." DST and ICMR sponsored national conference organized by **School of Pharmaceutical Sciences, Bahra University, Waknaghat, Shimla Hills, Solan, India**, September 29-30th 2016.